Universidad Autónoma de Madrid
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Distribución y degradación de las cianotoxinas microcistina y cilindrospermopsina en embalses

Distribution and degradation of the cyanobacterial toxins microcystin and cylindrospermopsin in reservoirs

Tesis Doctoral
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The job of collecting data is rarely done well enough unless it is animated by the prospects of theoretical interpretation, while such interpretation is almost always likely to be best done by someone who knows the feel of a wire or a rope when a messenger is running down to trip a water bottle.

G.E. Hutchinson, 1963

Si quisiera definir en una frase a los seres humanos, ésta podría valer: son animales que se creen las historias que ellos cuentan sobre sí mismos. Son animales crédulos

Mark Rowlands, 2008

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"*Yours is the first face that I saw. Think I was blind before I met you. I don't know where I am, I don't know where I've been, but I know where I want to go"* 4!

Indice / *Index*

Abreviaturas / *Abbreviations*

α: Coeficiente de absorción / *absorption coefficient*

ADDA: Ácido 3 amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienoico / *3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid*

ANOVA: Análisis de la varianza / *analysis of variance*

ANTX-A: Anatoxina-a / *anatoxin-a*

BMAA: β-N-metilamino-L-alanina / *β-N-methylamino-L-alanine*

chl *a:* Clorofila a / *chlorophyll a*

CYN: Cilindrospermopsina / *cylindrospermopsin*

DIC: Carbono Inorgánico Disuelto / *dissolved inorganic carbon*

DOC: Carbono orgánico disuelto / *Dissolved organic carbon*

EDTA: Ácido etilendiaminotetraacético / *ethylenediaminetetraacetic* acid

HAB: Afloramiento de algas dañinas / *harmful algal bloom*

HPLC: Cromatografía líquida de alta resolución / *high perfomance liquid chromatography*

IM: Materia inorgánica / *Inorganic matter*

JC I: Parque de Juan Carlos I

K_d: coeficiente de atenuación / *attenuation coefficient*

LC: Cromatografía líquida / *liquid chromatography*

LD_{50}: Dosis letal 50 / *Lethal dose 50*

LPS: Lipopolisacáridos / *lipopolysaccharides*

MC: Microcistina / m*icrocystin*

MC-XZ: Microcistina con ocupación de las posiciones variables 4 y 7 por los aminoácidos X y Z / *microcystin in which variable positions 4 and 7 are occupied by aminiacids X and Z*

MS: Espectrometría de masas / *mass spectrometry*

OC: Carbono orgánico / *organic carbon*

OM: Materia orgánica / *organic matter*

PAR: Radiación fotosintéticamente activa / *photosynthetically active radiation*

PDA: Detector de haz de diodo / *photodiode array detector*

PPA: Ensayo de inhibición de la actividad fosfatasa / *protein phosphatase inhibition assay*

S: sensibilidad del modelo / *model sensitivity*

SPE: Extracción en fase sólida / *solid phase extraction*

TFA: ácido trifluoroacético / *trifluoracetic acid*

UV-A: radiación ultravioleta A / *ultraviolet A radiation*

UV-B: radiación ultravioleta B / *ultraviolet B radiation*

UVR: radiación ultravioleta / *ultraviolet radiation*

WHO: Organización Mundial de la Salud / *World Health Organisation*

$z_{x\%}$: profundidad de la columna de agua a la que llega un x% de la radiación en superficie / *depth of the watercolumn which is penetrated by x% of surface irradiance*

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1. Introducción

1.1. Las cianobacterias y su potencial tóxico

1.1.1. Cianobacterias: Organización celular y ultraestructura

Las cianobacterias son procariotas fotoautótrofos oxigénicos que han logrado un notable éxito evolutivo (Whitton and Potts, 2000). El material genético se encuentra en la parte central del protoplasto, formando un único cromosoma circular, el cual ha sido secuenciado ya en algunas cepas (por ejemplo Kaneko et al., 1996). Asímismo, el citoplasma de cianobacterias está ocupado, principalmente en las zonas periféricas, por tilacoides que albergan el aparato fotosintético. La coloración característica de las cianobacterias, que dio lugar al término algas verde-azuladas, se debe a una peculiaridad de este sistema fotosintético: la presencia de pigmentos específicos junto a la clorofila *a*. Estos pigmentos, denominados genéricamente ficobilinas, incluyen entre otras a las ficocianinas y a las ficoeritrinas y permiten el aprovechamiento de radiación en longitudes de onda no cubiertas por clorofilas o carotenoides (fig. 1.1).

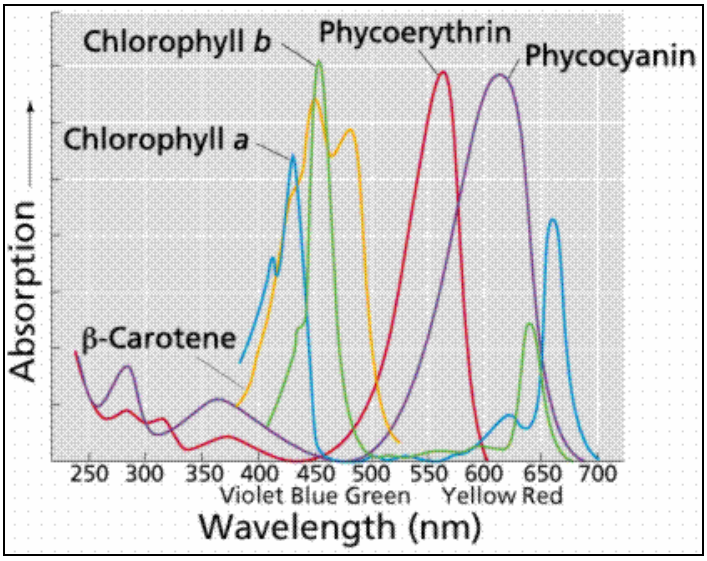

Figura 1.1: Espectro de absorción de los pigmentos fotosintéticos (fuente: dwb.unl.edu)

El interior celular de algunas cianobacterias puede albergar también otro tipo de estructura fundamental a la hora de garantizar un óptimo aprovechamiento de las condiciones ambientales: las vesículas de gas, inclusiones citoplasmáticas que pueden llenarse de gas. Esto permite regular la densidad de las células, con ello se variará su

flotabilidad y se podrá regular la posición del organismo en la columna de agua. Las vacuolas de gas, con una densidad equivalente aproximadamente a una cuarta parte de la densidad del agua, actúan favoreciendo la flotación. Dicha flotación será compensada con determinados productos del interior celular, principalmente los carbohidratos, que ejercerán de lastre. De esta forma, si asumimos una situación de nutrientes suficientes, la flotación dependerá directamente de la radiación recibida. Con escasa radiación, el balance se desplazará hacia el consumo de carbohidratos, con lo que la densidad celular baja y el organismo asciende en la columna. Sin embargo, con suficiente irradiancia recibida, el organismo producirá importantes acúmulos de carbohidratos que lo lastrarán hacia zonas más profundas, de menor irradiancia, pero más ricas en nutrientes, de los cuales podrá reabastecerse. Esta capacidad, presente en algunas especies, ha dado lugar a un movimiento diario finamente ajustado, pudiendo migrar dichas especies a lo largo de la columna de agua, aprovechando al máximo los recursos energéticos y los nutrientes disponibles (Walsby, 1987, Ibelings et al., 1991, Rabouille, 2005).

Otra característica importante de determinadas cianobacterias es su capacidad de fijar nitrógeno atmosférico. De hecho, las Nostocales y Stigonematales han desarrollado estructuras específicas, los heterocistos, capaces de generar un ambiente microanaerobio que permite que en su interior las enzimas nitrogenasa reduzcan el nitrógeno (N_2) a amonio (NH_4^+), el cual así puede ser incorporado (Bryant et al., 1994). A pesar de haberse relacionado tradicionalmente la fijación de nitrógeno con la existencia de estas células diferenciadas, se ha demostrado en numerosas ocasiones que dicha fijación puede darse también sin la formación expresa de heterocistos (Fernández Valiente et al., en prensa). Incluso, las cianobacterias se han considerado unos de los principales responsables de la aportación de nitrógeno en océanos (ej.: Kitajima et al., 2009). Otro tipo de célula diferenciada que algunos géneros filamentosos de cianobacterias pueden producir, y que tienen también connotaciones taxonómicas, son los acinetos. Se trata de células de resistencia, de morfología distinta a las células vegetativas, que en su interior acumulan compuestos de reserva, principalmente gránulos de cianoficina y glucógeno. Su formación se ha relacionado con situaciones de limitación en nutrientes o energía (Sili et al., 1994), si bien también se subraya la necesidad de unas condiciones mínimas en el medio que permitan mantener funciones fisiológicas básicas (Fay et al., 1984; Moore et al., 2003). Bajo determinadas condiciones, los acinetos podrán germinar y dar lugar a nuevos filamentos (ej.: Adams and Duggan, 1999; Moore et al., 2003).

1.1.2. Taxonomía de Cianobacterias

La presencia, las características y la posición de heterocistos y acinetos son algunas de las herramientas utilizadas en la clasificación taxonómica de cianobacterias, una clasificación que – como en muchos otros procariotas – es problemática y se encuentra sujeta a continua revisión. Siguiendo una aproximación botánica (p.e. Komarek y Anagnostidis, 1989), podemos establecer cinco grandes grupos de cianobacterias, grupos que encuentran también sus equivalentes en la clasificación bacteriológica (Rippka et al., 1979). Basándonos en Castenholz y Waterbury (1989) y Whitton y Potts (2000) se puede establecer la siguiente caracterización de dichos grupos:

Filamentosas		**Ejemplo de géneros potencialmente tóxicos**
Oscillatoriales	Filamentosas sin ramificaciones (puede incluir falsas ramificaciones); reproducción por división binaria, sin heterocistos ni acinetos descritos	*Planktothrix, Phormidium, Lyngbia*
Nostocales	Crecimiento similar a las Oscillatoriales; forman heterocistos; algunas especies presentan acinetos	*Anabaena, Aphanizomenon, Cylindrospermopsis, Nodularia*
Stigonematales	Crecimiento similar a Oscillatoriales, pero pudiendo presentar ramificaciones; forman heterocistos; algunas especies presentan acinetos	*Hapalosiphon, Umezakia*
No filamentosas		
Chroococcales	Unicelulares o agregados no filamentosos unidos por pared o mucílago; división binaria en uno, dos o tres planos, de forma simétrica o asimétrica, puede reproducirse también por gemación, aparición poco frecuente de células de superviviencia	*Microcystis, Snowella, Woronichinia*
Pleurocapsales	Unicelulares o agregados no filamentosos unidos por pared o mucílago; las células se reproducen por división interna en células hijas de menor tamaño o por este método y fisión binaria, aparición poco frecuente de células de superviviencia	A caracterizar

Tabla 1.1: Órdenes de cianobacterias y ejemplos de géneros tóxicos

1.1.3. Ecología de las cianobacterias

Los registros más antiguos de organismos aparentemente ligados a las actuales cianobacterias datan de los mismos albores de la vida en el planeta. El caso más antiguo, y muy estudiado, hace referencia a los fósiles del Apex Chert, descubiertos en Australia Occidental (Schopf, 2000). La antigüedad de estos fósiles, atrapados por calor y presión en una cama de arena, limo y lodo, ha sido estimada en torno a los 3500 millones de años. En estos registros se descubrieron multitud de restos petrificados de organismos cuyas dimensiones y formas coinciden con cianobacterias actuales (fig. 1.2). Otro yacimiento de importancia es el Gunflint Chert en el Lago Erie (EEUU), con una antigüedad estimada de 2090 millones de años. Es de destacar que los yacimientos de piedras ricas en hierro cercanos a fósiles de este tipo mostraban la presencia de óxidos de hierro, lo cual indica la presencia de oxígeno fotosintético en una atmósfera por aquel entonces aún anaerobia (Klein y Buekes, 1992).

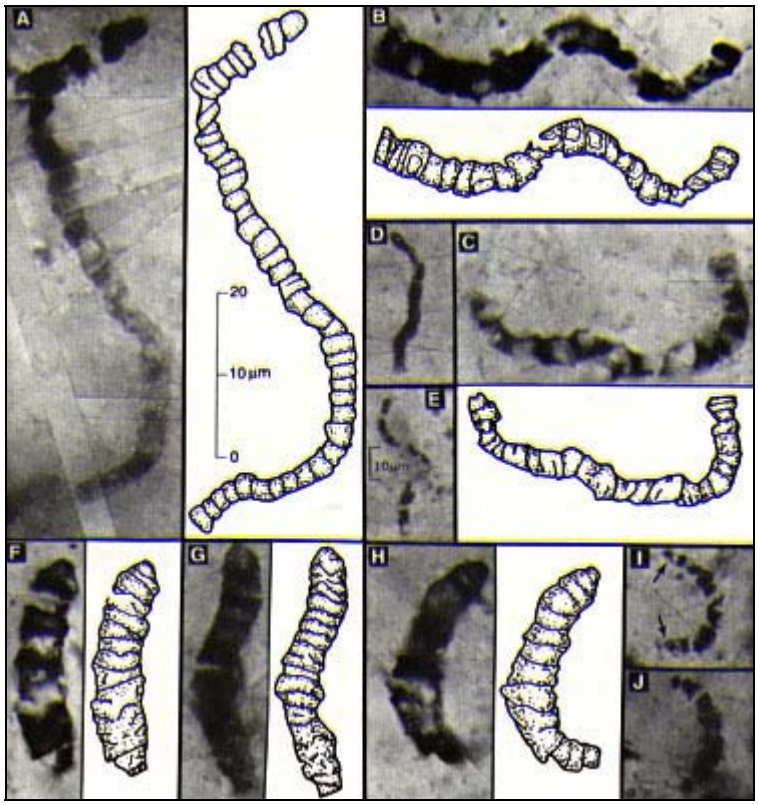

Figura 1.2. Fósiles similares a cianobacterias filamentosas del Apex Chert (Schopf 2000))

Desde los comienzos, las cianobacterias han estado fuertemente representadas en el planeta y en muchas ocasiones, especialmente en la antigüedad, en forma de

estromatolitos. Aún en la actualidad, es posible observar algunos estromatolitos vivos, principalmente en la zona de Australia Occidental – cercana a la zona de descubrimiento del Apex Chert – o en zonas del Caribe. Si bien los estromatolitos modernos escasean, no podemos decir lo mismo de las cianobacterias no formadoras de este tipo de estructuras. Hoy en día podemos encontrar cianobacterias en prácticamente cualquier sistema húmedo (Whitton y Potts, 2000), incluyendo ambientes extremos como, por ejemplo, las regiones polares, aguas salinas y salobres, los desiertos más cálidos (Wynn-Williams, 2000) o aguas termales (Ward and Castenholz, 2000).

Sin embargo, la mayoría de géneros se decanta por sistemas menos extremos, habiendo colonizado ambientes terrestres de elevada humedad, habiendo formado relaciones simbióticas con muy diversos organismos y siendo muy habituales en mares, lagos, ríos y embalses de todo el mundo. En estos sistemas es cada vez más habitual encontrar crecimientos masivos y repentinos de cianobacterias, los denominados "blooms" o afloramientos. En zonas templadas, éstos aparecen generalmente en época estival. En tal caso, la sucesión típica establecida para sistemas profundos sería la de un crecimiento inicial de diatomeas en primavera, seguido de un fuerte desarrollo de algas verdes coincidiendo con las condiciones óptimas de nutrientes. Finalmente, con temperaturas máximas y un paulatino agotamiento de nutrientes se dispararían las cianobacterias (Reynolds, 1984, Sommer et al., 1989; Oliver y Ganf, 2000). Este crecimiento en la época más cálida no ha de relacionarse con una preferencia de las cianobacterias por temperaturas elevadas, hipótesis de hecho muy discutible, sino más bien con las ventajas que columnas de agua bien estratificadas suponen para estos organismos (Oliver and Ganf, 2000). Dicha columna de agua estabilizada permite a las cianobacterias aprovechar al máximo su capacidad de regular su flotación y con ello su ubicación en el gradiente vertical, buscando las zonas óptimas para la fotosíntesis y la captación de nutrientes (Ganf y Oliver, 1982, Steinberg and Hartmann, 1988). Algunas especies de cianobacterias, por ejemplo *Microcystis aeruginosa*, son capaces de llevar esta apuesta por su flotación hasta los extremos de formar natas o "scums" que flotan sobre la superficie del agua como si de pintura verde se tratara (foto 1.1), lo que sólo es posible gracias a un fortísimo movimiento ascendente, cuya velocidad aumentará en principio con el tamaño de las colonias (Oliver and Ganf, 2000).

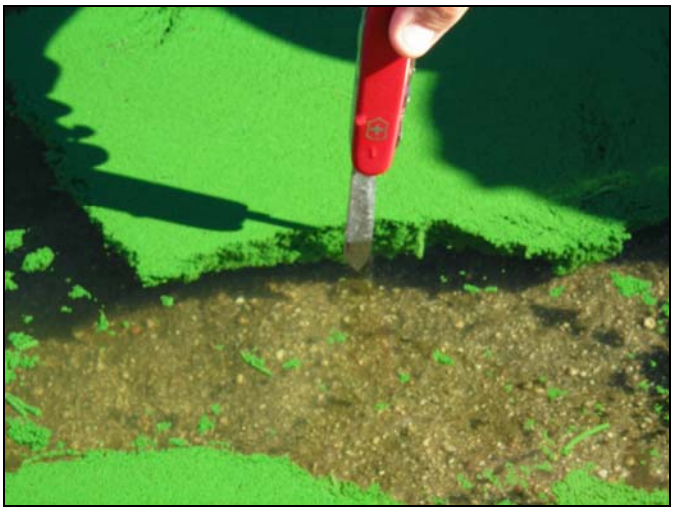

Foto 1.1: Acumulación de Microcystis en el embalse de Cogotas

La anteriormente descrita flotación de cianobacterias se verá influenciada de manera directa por la irradiación, pero también la propia competencia por dicha radiación será un elemento definitivo a la hora de evaluar las posibilidades de éxito de las cianobacterias. En su favor juega su amplia dotación en pigmentos, la cual permite el aprovechamiento de prácticamente cualquier longitu de onda en el rango de la radiación fotosintéticamente activa (Glazer, 1982; Glazer et al., 1994). La composición espectral de la irradiancia varía fuertemente en profundidad según las características de la columna de agua, con lo que la capacidad de respuesta de las cianobacterias a este cambiante perfil de irradiancia recibida resulta fundamental. Por ejemplo, las algas verdes absorberán gran cantidad de irradiancia, permitiendo el paso de radiación naranja-verdosa, para la cual no disponen de pigmentos (Kirk, 1983). Esta radiación será, sin embargo, aprovechada por las cianobacterias. Por contra, cuando dominan las cianobacterias, secuestrarán prácticamente todo el espectro de radiación, agotando en buena medida el recurso lumínico. Esto provocará condiciones de baja irradiancia para gran parte de la columna de agua, condiciones en las que, por lo general, se ha asumido un mejor crecimiento de cianobacterias, dado su menor consumo basal de energía (Mur, 1983; Chorus and Bartram, 1999).

Por lo demás, las condiciones idóneas para la formación de blooms son objeto aún de controversia y no han podido esclarecerse por completo. Factores que se han sugerido que pudieran favorecer el desarrollo de comunidades de cianobacterias son una mayor eficiencia a la hora de captar elementos traza (Hyenstrand et al., 1998) o una

mayor resistencia a la predación, por ejemplo gracias al mayor tamaño de las colonias o fascículos que pueden formar (Kamjunke et al., 1996). Los nutrientes nitrógeno (N) y fósforo (P) suelen ser los principales estimulantes del crecimiento de algas o cianobacterias (Reynolds y Walsby, 1975), en tanto que la demanda de estos suele ser mayor que la disponibilidad habitual. La cantidad, proporción y composición química de estos nutrientes han sido relacionadas con la magnitud, duración y composición de los afloramientos (Paerl, 2008). En el caso de las cianobacterias, el establecimiento de ratios N:P que pudieran favorecer el desarrollo de éstas ha sido objeto de estudio desde hace décadas. Smith (1983) sugirió que con relaciones bajas (<15) de N:P, tanto disueltas como totales, las cianobacterias podían verse favorecidas. Ratios mayores (>20) potenciarían la dominancia de algas eucariotas. Estas relaciones, si bien no definitivas, parecen cumplirse bastante bien en algunos sistemas de zonas tropicales, subtropicales y templadas, con estratificación estival y largos tiempos de residencia (Downing et al., 2001). Sin embargo, existen también numerosas ocasiones en las que esta regla se ve incumplida (Oliver and Ganf, 2000; Paerl, 2008), por ejemplo, en escenarios de elevada carga de N y P, de forma que ambos dejan de ser limitantes y son otros factores, la flotabilidad, el aprovechamiento lumínico etc. los que pueden resultar definitivos. También tiempos de residencia suficientemente breves para evitar el – por norma – lento crecimiento de cianobacterias pueden afectar a esta regla general.

Mención aparte merecen las cianobacterias fijadoras de nitrógeno. Éstas resultan favorecidas cuando las fuentes de nitrógeno inorgánico son limitantes (Oliver y Ganf, 2000). Asimismo, estas especies fijadoras pueden llegar a suponer una fuente adicional de nitrógeno, contribuyendo a su vez al paulatino enriquecimiento en nitrógeno de las aguas afectadas (Paerl, 1988), y permitiendo la presencia simultánea de cianobacterias fijadoras y no fijadoras (Paerl, 1990) De ahí, que sea habitual observar la presencia simultánea de géneros fijadores como *Aphanizomenon* o *Anabaena* y no fijadores como *Microcystis*. Por otra parte, las cianobacterias muestran una relación peculiar con el fósforo. En bajas concentraciones de este nutriente (< 10 μg l^{-1}) su crecimiento se ve muy limitado (Cooke et al., 1993). Sin embargo, a pesar de estas limitaciones, en condiciones de baja concentración de fósforo los diversos géneros cianobacterianos parecen haber desarrollado eficaces estrategias. *Microcystis*, por ejemplo es capaz de tomar fosfato del ambiente muy rápidamente y almacenar gran cantidad del mismo

(Kromkamp et al., 1989), lo cual, aprovechando la capacidad de regular su flotación, le convierte en un excelente acumulador de P en zonas profundas.

En todo caso, y a pesar de las dudas que aún existen en cuanto a las condiciones ambientales que favorecen a las cianobacterias frente a otros géneros fitoplanctónicos, parece evidente que los afloramientos de organismos fotoautótrofos se ven beneficiados por el paulatino enriquecimiento en nutrientes que la actividad humana ha causado en muchos sistemas. Y es que a pesar de que las proliferaciones de cianobacterias parecen haberse dado también en tiempos lejanos, la frecuencia y magnitud de estos fenómenos se ha visto fuertemente incrementada, seguramente gracias al gran aporte antropogénico de nutrientes. Quizás esta eutrofización de las aguas sea el elemento estructural que permite el mayor y más frecuente desarrollo de blooms, mientras que el hecho de que dichos blooms estén protagonizados por cianobacterias u otros grupos del fitoplancton se deba más a determinados condicionantes puntuales.

En el ámbito de estos lagos y embalses – indispensables para la actividad humana y a su vez sometidos a fuertes impactos por parte de dicha actividad humana – una peculiaridad de las cianobacterias se hace especialmente notable: su capacidad para producir diversos compuestos tóxicos, las cianotoxinas.

1.1.4. Tipos de cianotoxinas

El término cianotoxina es tan amplio como parece indicar, pues engloba todos aquellos compuestos nocivos que son producidos mediante el metabolismo secundario de cianobacterias. Por otra parte, a pesar del gran número de estudios dedicados a las cianotoxinas, todavía no se ha determinado cuál es su verdadera función ecológica. Entre sus funciones se han propuesto: la defensa ante el herbivorismo (Lampert, 1981); efectos alelopáticos sobre otros competidores algales (Czarnecki et al, 2006); señalización intra- o intercelular (Dittmann et al, 2001a); quelantes de hierro (Utkilen 1995), etc.

Las distinas cianotoxinas son compuestos químicamente muy diversos, pero aún así es posible establecer dos grandes grupos: alcaloides y péptidos. Dentro de los alcaloides tóxicos hemos de mencionar en primer lugar el grupo de las neurotoxinas, que engloba anatoxina-a, homoanatoxina-a, anatoxina-a(s) y saxitoxinas. Ambas

anatoxinas toman su nombre del género *Anabaena*, del cual fueron inicialmente aisladas. En el caso de la anatoxina-a y sus variantes, su actuación se centra en los receptores nicotínicos de acetilcolina, activando una estimulación continua del impulso nervioso, la cual puede traducirse finalmente en parálisis muscular y fallo respiratorio (Carmichael, 1979). La dosis letal 50 (LD_{50}) oral en ratón para este compuesto ha sido estimada en más de 5 mg/kg de peso corporal, mientras que una administración intraperitonal rebaja este valor a 250 μg/kg (Fitzgeorge et al., 1994). El efecto de la anatoxina-a(s) consiste en el bloqueo de la actividad acetilcolinesterasa, de manera similar a la actuación de diversos insecticidas organofosforados, la LD_{50} intraperitonal se sitúa en 20 μg/kg (Mahmood y Carmichael, 1986). Las saxitoxinas, conocidas también como PSPs (paralytic shellfish poison) son más conocidas por los episodios en que son producidas por dinoflagelados marinos (Steidinger, 1993), pero existen también diversas cianobacterias responsables de su síntesis. Por ejemplo, un bloom de *Anabaena circinalis* en el río Darling (Australia) fue responsable de la muerte de más de mil cabezas de ganado, así como de serios problemas en el suministro de agua de diversas localidades (Humpage et al., 1993, 1994). El efecto de la saxitoxina se centra en la membrana de los axones, bloqueando los canales de Na^{2+} y afectando con ello la correcta generación del impulso nervioso (Caterall, 1980), pudiendo llevar a parálisis y fallo respiratorio. En este caso, la LD_{50} oral en ratón para este compuesto ha sido estimada en 263 μg/kg de peso corporal, mientras que una administración intraperitonal rebaja este valor a 3,4 μg/kg (Stephenson, 1960).

Como ya se ha comentado, las saxitoxinas son producidas principalmente en aguas marinas por dinoflagelados, y la presencia debida a cianobacterias es bastante limitada y poco estudiada. Sin embargo en el caso de la anatoxina-a, ha sido descrita en aguas por ejemplo de Irlanda (James et al., 1997), Alemania (Bumke-Vogt et al., 1999) o Corea (Park et al., 1998). Por contra, en España, su presencia ha sido mínima en el pasado (Carrasco et al., 2007)

Además de estos alcaloides neurotóxicos, hemos de mencionar otro alcaloide de pequeño tamaño: la cilindrospermopsina, cuya denominación procede de *Cylindrospermopsis raciborskii*. Si bien inicialmente se consideró que la cilindrospermopsina actuaba sobre el hígado, pronto se descubrió que el daño producido por este alcaloide era mucho más amplio, afectando órganos como riñón, corazón,

glándulas adrenales, sistema vascular y linfático (Falconer and Humpage, 2006). La cilindrospermopsina ha sido considerada tradicionalmente típica de regiones cálidas, habiendo sido descrita muy frecuentemente en Oceanía (ej.: Shaw et al., 1999), pero también en zonas como Israel (Banker et al., 1997), Florida (Chapman and Schelske, 1997) o Brasil (Bouvy et al., 2000). Sólo recientemente esta toxina está empezando a observarse en climas más templados. De hecho, la primera presencia masiva de organismos productores de cilindrospermopsina en aguas europeas fue descrita en 2004 en el embalse de Arcos, Cádiz (Quesada et al., 2006). Desde entonces, han sido cada vez más frecuentes las observaciones de dicha toxina, habiendo sido detectada, por ejemplo, en 102 de los 115 puntos muestreados por Rücker et al. (2007) en Alemania.

Finalmente, cabe mencionar el grupo de cianotoxinas más estudiado y responsable de un mayor número de casos de intoxicación, los péptidos hepatotóxicos microcistina y nodularina, nombrados una vez más según los primeros organismos productores descritos, *Microcystis* y *Nodularia* respectivamente. Ambas se caracterizan por la presencia de un aminoácido muy poco corriente, el ácido 3 amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienoico (ADDA) y por centrar el daño producido en el sistema hepático (Dawson, 1998). En España estas toxinas han sido las más habituales durante los últimos años (Carrasco et al., 2006)

Aún no habiéndolos incluido en los dos grupos de cianotoxinas descritos anteriormente, hemos de mencionar en este punto a los lipopolisacáridos (LPS) de las paredes celulares de cianobacterias. Estos compuestos parecen poder ser considerados responsables de irritación y reacciones alérgicas, si bien aparentemente en menor grado que los LPS de otras bacterias Gram negativas (Raziuddin et al., 1983), aunque existen también trabajos que sugieren una toxicidad similar de LPS de cianobacterias y bacterias heterotróficas(Bernardova et al., 2008). Pero sobre todo es significativo el hecho de que estos LPS pueden influir en la toxicidad de otros compuestos (Pietsch et al., 2001; Rapala et al., 2002, Lindsay et al., 2006). Finalmente, queremos mencionar brevemente el caso del aminoácido BMAA (β-N-metilamino-L-alanina). Inicialmente este compuesto se encontró en semillas de cicadeáceas en la isla de Guam, y se ha relacionado con la aparición de esclerosis lateral amiotrófica (ELA) y Parkinson (Murch et al., 2004a). También, se ha descrito la presencia de BMAA en tejido cerebral de personas afectadas por Alzheimer en Canadá (Murch et al., 2004a,b). Aparentemente, el

BMAA se ve sujeto a procesos de biomagnificación, este proceso quedó evidenciado por Cox et al. (2005) al identificar que la alta incidencia de ELA en los habitantes nativos de la isla de Guam podía deberse a que consumen murciélagos, los cuales a su vez son capaces de acumular gran cantidad de BMAA. Dicha magnificación se ve agravada gracias a la incorporación del BMAA a estructuras peptídicas o protéicas, lo que permite una más lenta liberación de la toxina (Murch et al., 2004b). El nexo con las cianobacterias se establece cuando se observa que *Nostoc,* viviendo simbióticamente con las cicadeáceas, es el auténtico responsable de la síntesis de BMAA, el cual es luego acumulado en las semillas de la planta. Desde entonces, el BMAA ha sido descrito, por ejemplo, en las doce muestras de afloramientos, acumulados y tapetes de cianobacterias analizadas en Reino Unido (Metcalf et al., 2008). En todo caso, aún se trabaja en la puesta a punto de métodos analíticos completamente fiables para este producto, dado que por ejemplo es fácilmente confundible con el ácido α-,γ-diaminobutírico.

El ejemplo del BMAA nos muestra que nuestro conocimiento acerca de las cianotoxinas es aún tremendamente limitado. El creciente interés por estos compuestos, la mejora de las técnicas analíticas, así como un mayor control de los cuerpos de agua en todo el mundo nos llevará seguramente al descubrimiento de nuevas sustancias, tóxicas o quizás beneficiosas, producidas por estos organismos. En este sentido, se estudió la presencia de péptido sintetasas no ribosomales en la colección de cianobacterias del Instituto Pasteur (Christiansen et al., 2001). El resultado demostró que más del 75% de las cepas presentaba actividad de estos compuestos, lo cual nos da a entender la enorme capacidad de síntesis de compuestos bioactivos que tienen las cianobacterias, en este caso en forma de péptidos no ribosomales.

En los estudios que aquí presentamos, hemos optado por centrar nuestra atención en dos cianotoxinas en concreto, las microcistinas y la cilindrospermopsina. Esta decisión se ha debido a la predominancia de las primeras y la novedad de la segunda, al hecho de representar los dos grandes grupos de cianotoxinas (peptídico y alcaloides) y también a nuestra hipótesis de que su comportamiento en el sistema puede ser radicalmente diferente, permitiéndonos mostrar lo complejo y variado de sus dinámicas una vez presentes en el sistema.

1.1.5. Organismos productores de cianobacterias

A la hora de evaluar la potencialidad tóxica de una determinada especie de cianobacterias, habremos de enfrentarnos a la dificultad que supone que la capacidad de síntesis de cianotoxinas no se encuentre regulada a nivel de género o especie, sino que se expresa a nivel de cepa. De esta manera, no se puede establecer un listado de especies o géneros tóxicos. En todo caso, se podrán considerar como especies potencialmente tóxicas aquellas en las que alguna cepa ha producido una determinada toxina. En este sentido, la observación de muchos episodios tóxicos, y el aislamiento de diversas cepas alrededor del mundo nos permite tener una idea aproximada del potencial tóxico de algunos de los géneros cianobacterianos. La tabla 1.2 recoge la producción de las distintas cianotoxinas por diversas especies de cianobacterias planctónicas, basándonos en la tabla construida por Falconer (2005), completando la información con datos encontrados en Fristachi y Sinclair (2008) y en la literatura disponible.

Especie	Toxina	Ejemplo de aparición geográfica
Anabaena bergii	Cilindrospermopsina	Australia
Anabaena circinalis	Microcistina	Francia
Anabaena circinalis	Saxitoxina	Australia
Anabaena flos-aquae	Anatoxina-a	Canadá, Alemania
Anabaena flos-aquae	Anatoxina-a(s)	Canadá
Anabaena flos-aquae	Microcistina	Canadá y Noruega
Anabaena lapponica	Cilindrospermopsina	Finlandia
Anabaena lemmermannii	Anatoxina-a(s)	Dinamarca
Anabaena lemmermannii	Microcistina	Noruega
Anabaena lemmermannii	Saxitoxina	Finlandia
Anabaena planktonica	Anatoxina-a	Italia
Anabaena variabilis	BMAA	EE.UU
Anabaenopsis millerii	Microcistina	Grecia
Aphanizomenon flos-aquae	Saxitoxina	EE.UU
Aphanizomenon flos-aquae	Anatoxin-a(s)	Canadá
Aphanizomenon flos-aquae	**Cilindrospermopsina**	**Alemania**
Aphanizomenon gracile	LPS	EE.UU
Aphanizomenon issatschenkoi	**Anatoxina-a**	**Nueva Zelanda**
Aphanizomenon issatschenkoi	Saxitoxina	EE.UU
Aphanizomenon lemmermannii	Saxitoxina, anatoxina-a(s)	Portugal, Dinamarca

Aphanizomenon ovalisporum	Cilindrospermopsina	Israel, Australia
Aphanizomenon sp.	Anatoxina-a	Finlandia, Alemania
Arthrospira fusiformis	Microcistina	Africa, España
Cylindrospermum sp.	Anatoxina-a	Finlandia
Cylindrospermopsis raciborskii	Cilindrospermopsina	Australia, Tailandia, EE.UU
Cylindrospermopsis raciborskii	Saxitoxinas	Brasil
Cylindrospermopsis raciborskii	BMAA	
Cylindrospermopsis raciborskii	otras	Francia, Alemania, Portugal
Lyngbya wollei	Saxitoxinas	EE.UU.
Microcystis aeruginosa	Microcistina	Sudáfrica, Australia, Japón, Reino Unida, EE.UU
Microcystis botrys	Microcistina	Dinamarca
Microcystis ichthyoblabe	Microcistina	República Checa
Microcystis flos-aquae	**Microcistina**	**España**
Microcystis viridis	Microcistina	Japón
Nodularia spumigena	Nodularina	Mar Báltico, Australia
Nostoc sp.	Microcistina	Finlandia, Reino Unido
Planktothrix agardhii	Microcistina	Finlandia, China
Planktothrix agardhii	BMAA	
Planktothrix flavosum	Anatoxina-a	Francia
Planktothrix formosa	Homoanatoxina-a	Noruega
Planktothrix mougeotii	Microcistina	Dinamarca
Planktothrix rubescens	Microcistina	Noruega, Alemania
Prochlorococcus marinus	BMAA	Mar de los Sargazos
Raphidiopsis curvata	Cilindrospermopsina	China
Raphidiopsis mediterranea	**Anatoxina-a, homoanatoxina-a**	**Japón**
Snowella lacustris	Microcistina	Noruega
Synechococcus sp.	BMAA	EE.UU
Trichodesmium thiebautii	BMAA, Saxitoxina	Mar de los Sargazos
Umezakia natans	Cilindrospermopsina	Japón
Woronochinia naegeliana	Microcistina	Dinamarca

Tabla 1.2: Especies de cianobacterias planctónicas en las que se ha observado la producción de cianotoxinas y ejemplos de aparición según Falconer, 2002. Se han añadido entradas en base a la tabla encontrada en Fristachi y Sinclair, 2008 (subrayado) y la literatura disponible (negrita)

Esta tabla muestra lo ampliamente distribuida que está la capacidad de producir metabolitos potencialmente tóxicos entre las cianobacterias. A su vez, la rápida incorporación de nuevas especies potencialmente tóxicas muestra que la mayor concienciación y la mayor disponibilidad de técnicas analíticas permiten una cada vez mejor comprensión de este fenómeno global. Los casos específicos de la microcistina y cilindrospermopsina serán tratados más adelante.

1.1.6. Afloramientos de cianobacterias tóxicas: Ocurrencia y mitigación

Dicho carácter global de los afloramientos de cianobacterias tóxicas ha sido descrito por Carmichael (2008), quien reunió datos correspondientes a todo el mundo (fig 1.3), mostrando la gran cantidad de países en los que se han descrito ya blooms tóxicos. Las más evidentes ausencias se centran en el continente africano, seguramente no por falta de estos afloramientos, sino porque el estudio de estos episodios no ha recibido una atención preferente en estas regiones.

Figura 1.3. Aparición de blooms de cianobacterias tóxicas. Los puntos identifican a los países en que se ha registrado algún episodio (Carmichael, 2008)

Es interesante también observar otro apunte aportado por Carmichael (2008) en cuanto a la literatura científica dedicada al fenómeno de los afloramientos tóxicos (fig. 1.4). Desde los años 60, el incremento exponencial de publicaciones referentes a este tema indica posiblemente una mayor incidencia de blooms tóxicos debido a la decreciente calidad de las aguas, pero también un creciente interés de científicos y gestores en la amenaza que suponen las cianobacterias tóxicas.

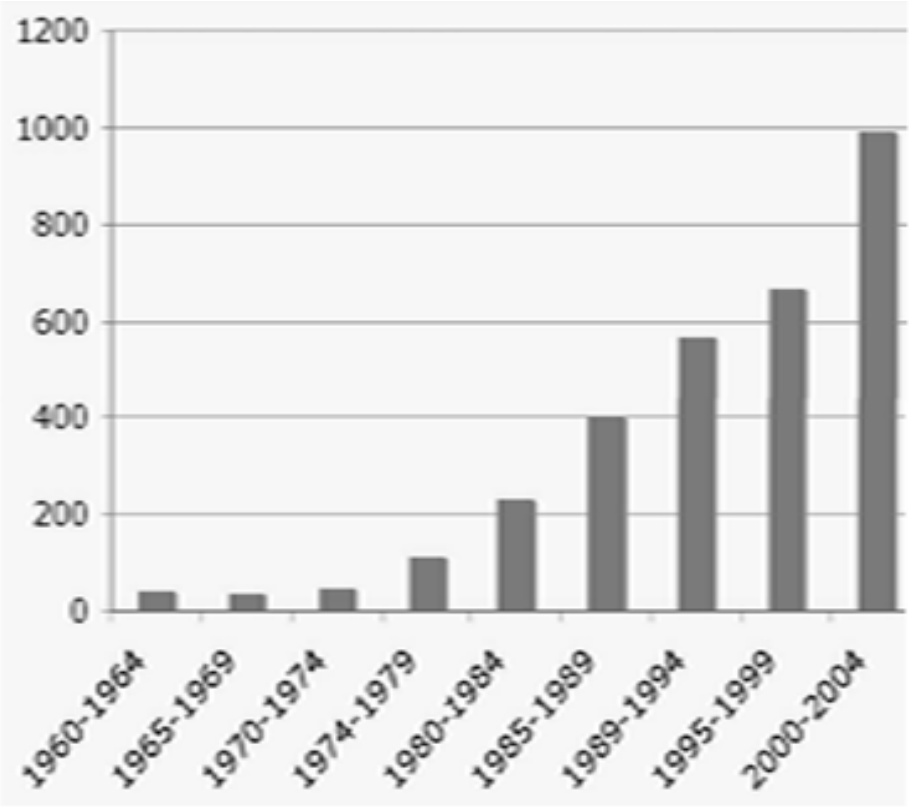

Figura 1.4. Número de publicaciones referidas a apariciones de blooms cianobacterias tóxicas (Carmichael, 2008)

Este mismo interés ha disparado la investigación en cuanto a posibles métodos que permitan mitigar o minimizar el riesgo de afloramientos de cianobacterias potencialemente tóxicas. Las estrategias encaminadas a tal fin se han centrado en la reducción de nutrientes, apostando principalmente por N y P ya que, como se ha comentado anteriormente, dado su carácter limitante suelen ser los mayores estimulantes del crecimiento de algas o cianobacterias (Reynolds and Walsby, 1975). Generalmente, el primer paso en estos trabajos consiste en reducir el aporte de fósforo, en tanto que su reducción dificulta cualquier desarrollo masivo de fitoplancton y desplaza el equlibrio N:P hacia valores más altos, en principio menos favorables a cianobacterias. Existen algunos trabajos exitosos en sistemas como, por ejemplo, el lago Washington en EE.UU. (Edmondson and Lehman, 1981) o el fiordo Himmerfjärden en Suecia (Elmgren and Larsson, 2002). Se ha observado también que en otras ocasiones, especialmente cuando ya existen acúmulos importantes de P en sedimento o gracias a otras fuentes naturales, es necesaria la reducción combinada de los aportes de N y P (Vollenweider and Kerekes, 1982). También para garantizar una mejora a medio y largo plazo suele ser necesaria la reducción de ambas variables (Paerl, 2008)

La aplicación de estos métodos de reducción de aporte de nutrientes a menudo no encuentra una respuesta inmediata. Esto se debe en buena medida a que muchos sistemas han logrado ya una inmensa acumulación de nutrientes. En ocasiones esto se puede deber a los propios blooms que, si no son consumidos, sedimentarán, creando un importantísimo reservorio de nutrientes dispuestos a ser liberados. De esta forma, un bloom pasado puede suponer el mejor aliciente para afloramientos futuros. Surge así la necesidad de controlar tanto las fuentes internas como externas de nutrientes. Algunas de las estrategias encaminadas a este fin son el secuestro y precipitación de P, la retirada de sedimento o la minimización de tiempos de residencia del agua en el sistema afectado (Paerl, 2008).

En todo caso, existen también voces que ponen en duda la viabilidad misma de estos métodos, apoyándose en teorías de "punto de no retorno". Un ejemplo de esto es el trabajo de Duarte et al. (2009) en el cual pone en duda que el cese de una presión antropogénica, como por ejemplo un excesivo aporte de nutrientes, pueda devolver un sistema a un estado previo a dicha presión. En palabras de los autores, y haciendo referencia al célebre "País de Nunca Jamás", "we submit that the expectation that ecosystems can be returned to an idealized past reference status by virtue of reducing direct human pressures is as likely as the existence of *Neverland*".

Desde hace tiempo se ha postulado que la recuperación de estados originales en lagos no es lineal, sino bastante compleja. Además, desde un punto de vista temporal, dicha recuperación suele no ser inmediata, sino que pueden observarse periodos de retraso de 10 o 15 años. Dicho comportamiento se ha explicado con factores como la presencia de reservorios internos, la necesidad de reajuste de cadenas tróficas, etc. (Jeppesen et al., 2005). Sin embargo, Duarte et al. (2009) van un poco más allá, postulando que cambios drásticos, superando un punto de no retorno, pueden llegar a alterar los valores de referencia mismos del sistema (fig. 1.5). Dicho cambio de sistema de referencia puede hacer imposible un retorno a condiciones previas al impacto, con la consecuente frustración para gestores y científicos. Es por ello que se defiende la necesidad de asumir la posibilidad de estos nuevos escenarios, para encaminar los esfuerzos de recuperación a alcanzar un sistema ecológico viable, aunque no sea coincidente con el ecosistema previo.

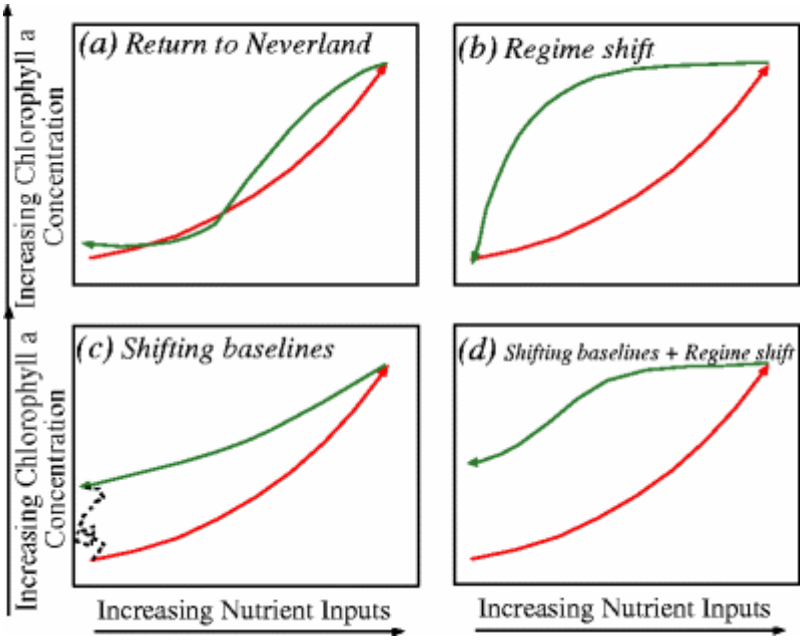

Figura 1.5: Posibles trayectorias en la recuperación de ecosistemas afectados por excesiva carga de nutrientes según Duarte et al. (2009). A) recuperación directa de condiciones previas B) trayectoria enrevesada volviendo a condiciones previas c) cambio de sistema de referencia d) cambio de sistema de referencia y trayectoria enrevesada, incapacidad de volver a condiciones iniciales

Además de los métodos de reducción de nutrientes, existen otras opciones que han sido puestas en funcionamiento de cara a la reducción del riesgo de afloramientos de cianobacterias potencialmente tóxicas. Algunas, por ejemplo, se han basado en el ciclo anual de las cianobacterias, especialmente en el caso de especies formadoras de acinetos y de *Microcystis*, centrándose en la reducción o eliminación del inóculo hibernante. Tales estrategias incluirían el dragado de sedimentos o la retirada de agua hipolimnética que pudiera contener poblaciones pelágicas hibernantes, y se han llevado a cabo con un elevado éxito en algunos casos (Poulickova, 1998). Otras estrategias se basarán en la ruptura física de la estratificación, lo cual podrá otorgar alivio puntual a la amenaza de determinados blooms cianobacterianos (Visser et al., 1996) o en el biocontrol mediante la introducción de peces depredadores que favorezcan al zooplancton y/o mediante plantas acuáticas que compitan por los nutrientes disponibles. A largo plazo, se trata de una estrategia que, por sí sola, no reúne unanimidad en cuanto a su utilidad (ej.: De Melo et al., 1992; Carpenter and Kitchell, 1992). Una desventaja adicional de estas estrategias, en relación a las cianobacterias, es el hecho de que no todas las especies fitoplanctónicas son consumidas de forma eficiente por el zooplancton. Las cianobacterias que forman grandes colonias o fascículos, así como las

cianobacterias filamentosas, son poco comestibles para el zooplancton, lo que dificulta el éxito de estas medidas y podría incluso tener efectos contraproducentes (McQueen, 1990; Ghadouani et al., 2003).

1.2. Las cianotoxinas

1.2.1. Microcistina

1.2.1.1. Estructura y tipos

El estudio de la microcistina se remonta bastante atrás en el tiempo, habiendo sido identificada su naturaleza peptídica ya en 1959 (Bishop et al., 1959). A esta primera caracterización le siguieron otras que se tradujeron finalmente en la obtención de la estructura química de cinco variantes – todas procedentes de *Microcystis aeruginosa* – por parte de Botes et al. (1984, 1985). Dicha estructura es la de un heptapéptido cíclico destacando el aminoácido ADDA (ácido 3 amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienoico) y dos posiciones que pueden ser ocupadas por distintos aminoácidos, posiciones aquí denominadas X e Y (fig. 1.6). Lo más habitual es que la posición X se vea ocupada por aminoácidos más hidrofóbicos, mientras que la posición Y es ocupada por otros más hidrofílicos. La ocupación variable de dichas posiciones, así como otras variaciones químicas, darán lugar a las distintas especies de microcistinas, las cuales se denominarán teniendo en cuenta el código de una letra de los aminoácidos en dichas posiciones. Estas distintas variantes mostrarán también distinto efecto tóxico, tal y como se detalla más adelante. El número de variantes descritas sigue aumentando paulatinamente. Por ejemplo, Codd et al. (2005) registraban la existencia de 71 variantes distintas.

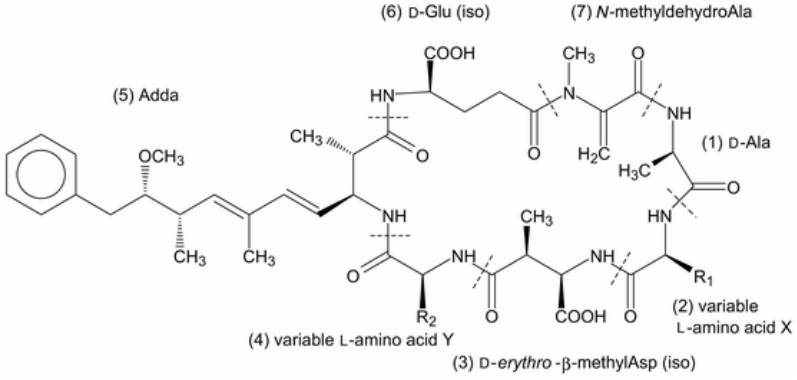

Figura 1.6. Estructura química genérica de la microcistina (www.rsc.org)

1.2.1.2. Síntesis y liberación

Para resolver las dudas acerca de la síntesis de las microcistinas, los primeros trabajos exitosos se centraron en identificar los sustratos utilizados en dicha síntesis. Marcando diversos sustratos con ^{13}C y suministrando éstos a *Microcystis aeruginosa*, Moore et al. (1991) pudieron observar el destino de estos sustratos en la estructura final de la toxina. Así por ejemplo, para el ADDA el organismo parecía recurrir a metionina, pero también a propionato, mientras que el acetato era pieza fundamental en la parte lineal de este aminoácido. Esta técnica fue aplicada para los distintos componentes del péptido, proponiendo los posibles precursores en gran detalle. Pero además de esto, se observó que las rutas de síntesis eran propias de L-aminoácidos, mientras que la mayoría de los aminoácidos presentes en la microcistina son D-aminoácidos, dicha racemización había de darse pues cuando el anillo peptídico estaba ya formándose.

Esta propuesta de precursores y rutas de síntesis debía verse confirmada encontrando los procesos biológicos que pudieran ser responsables. Ya en 1990, Kleinkauf y von Döhren habían observado que diversos antibióticos peptídicos eran producidos por medio de rutas no ribosomales, recurriendo a diversas enzimas multifuncionales. Considerando secuencias conservadas tanto en bacterias como en hongos que son capaces de producir otros péptidos no ribosomales (Borchert et al., 1992), fue posible identificar una serie secuencias que pudieran estar involucradas en la síntesis de peptido sintetasas en *Microcystis aeruginosa*. El siguiente paso fue el hallazgo de secuencias homólogas a las secuencias de péptido sintetasas de otros organismos y la comprobación de que estas secuencias aparecían sólo en organismos productores de microcistina. De esta manera se identificaron las primeras regiones involucradas en la síntesis de microcistina por vía no ribosomal, estas regiones fueron nombradas *mcyA, mcyB* y *mcyC* respectivamente (Meissner et al., 1996; Dittmann et al., 1996). Con posterioridad se describieron siete módulos más involucrados en distintos pasos de la síntesis de microcistina, recibiendo los nombres de *mcyD, mcyE, mcyF, mcyG, mcyH, mcyI* y *mcyJ* respectivamente y habiéndose establecido la función concreta de cada uno de ellos (Nishizawa et al., 1999, 2000; Tillett et al., 2000). Estos genes serán responsables de la síntesis de microcistinas al habilitar péptido sintetasas no ribosómicas que unen los distintos aminoácidos y poliquétido sintetasas, encargadas de la síntesis del ADDA. Cabe destacar que las rutas de síntesis propuestas en estos

trabajos son perfectamente coherentes con las rutas sugeridas por Moore et al. (1991) en base a los posibles precursores. La regulación de estos procesos biosintéticos de microcistina es aún una incógnita, habiendo sido propuesta la intensidad de la irradiancia como un posible activador de la transcripción de los genes *mcyB* y *mcyD*. Sin embargo, dicha activación no dio como resultado un mayor contenido celular de microcistina (Kaebernick et al., 2000).

Todos estos trabajos de caracterización de la ruta de síntesis y los genes involucrados fueron realizados sobre material biológico perteneciente a cepas del género *Microcystis*, concretamente *M. aeruginosa* PCC7806 en los trabajos de Tillet y *M. aeruginosa* K-139 en los de Nishizawa. Por ello, el siguiente objetivo fue la comprobación de la validez de estos hallazgos en otros géneros potencialmente productores. Christiansen et al. (2003) estudiaron a *Planktothrix agardhii*, encontrando componentes similares, pero con una organización distinta dentro del cluster genético. En el caso de *Anabaena*, sin embargo, las diferencias son tales que parecen indicar bien orígenes independientes de los respectivos genes o bien procesos de divergencia (Rouhiainen et al., 2004).

Considerando estas incógnitas acerca del origen y la evolución de los genes responsables de síntesis de microcistinas, resulta interesante comentar dos aspectos. Por un lado, Rantala et al. (2004) estudiaron la relación entre genes involucrados en el metabolismo primario y los genes *mcy*. Estos autores pudieron constatar su coevolución a lo largo de la historia evolutiva de las cianobacterias, sugiriendo la existencia de un juego de genes ancestrales de los cuales han surgido los actuales genes *mcy*. A su vez, tal y como apunta Falconer (2005), si situamos estos genes ancestrales en los orígenes de las cianobacterias, hace aproximadamente 2000 millones de años, nos daremos cuenta de que su existencia es previa a la de los eucariotas, la cual se estima comenzó hace unos 1500 millones de años. Esto indicaría que el fin de las microcistinas, al menos en su versión ancestral, no sería el de ser un compuesto tóxico para eucariotas.

Volviendo nuestra atención a la microcistina contemporánea, una vez producida la molécula, ésta tiende a permanecer en el interior celular, como parece ser propio de las cianotoxinas peptídicas, y es liberada tan sólo con la muerte y lisis de la célula productora. La limitada excreción de la toxina ha sido descrita por ejemplo por Sivonen (1990) en cultivos de *Oscillatoria* (*Planktothrix) agardhii*. Según la cepa estudiada, el

porcentaje medio de toxina disuelta era de menos del 10 o del 20%. También Rapala et al. (1997) y Wiedner et al. (2003), trabajando en *Anabaena* y *Microcystis* respectivamente, observaron la poca importancia de la fracción extracelular. Además, Rapala et al. (1997) mostraron que el aumento de la fracción extracelular parecía ser independiente de estímulos externos, aumentando sólo con el paso del tiempo, es decir, con el envejecimiento del cultivo y la lisis celular. Esta ausencia de procesos de excreción o pérdida de toxina intracelular ha sido confirmada también por Rohrlack y Hyenstrand (2007) trabajando con fuentes de carbono marcadas. Por el contrario, Ross et al. (2006) sugieren que la liberación de microcistina se incrementa en situaciones de estrés oxidativo. Esto sería más consecuente con los datos de Dittmann et al (2001b) acerca de la existencia de genes similares a los que codifican transportadores en bacterias dentro del cluster de genes responsables de la síntesis de microcistinas, o con los de Kabernick et al. (2001) acerca de la posible excreción activa de microcistinas bajo condiciones de alta irradiancia. Una vez en el medio, la microcistina es estable, no degradándose fácilmente por temperatura ni pH (Harada et al., 1996).

1.2.1.3. Efecto tóxico

El mecanismo tóxico de muchos compuestos se basa en la inhibición de una determinada actividad enzimática. Dicha inhibición será especialmente grave cuando reúne algunas características como son: una limitada disponibilidad de la enzima en el sistema celular, una baja afinidad no específica del compuesto tóxico por otros componentes celulares y la ubicación de la enzima afectada al comienzo de una ruta enzimática más compleja (Falconer, 2005). Estos criterios se ven cumplidos en el caso de la inhibición de enzimas fosfatasa por parte de las microcistinas. Estas enzimas son responsables de la eliminación de fosfatos en proteínas cuya actividad se regula justamente por la presencia o ausencia de dichos grupos fosfato (Barford, 1996). Por ejemplo, la presencia de fosfatos activa enzimas glucógeno fosforilasas, responsables de la ruptura de glucógeno en fosfato de glucosa, mientras que la presencia de fosfatos en las glucógeno sintetasas inhibe la génesis de glucógeno en base a fosfato de glucosa.

El desajuste entre enzimas fosfatasa y quinasa, responsables de transferir grupos fosfato, pueden afectar gravemente la estructura y función celular y ha sido relacionado con el desarrollo de células cancerígenas (Cohen and Cohen, 1989). Esto es coherente con los pioneros estudios de Falconer et al. (1988) que establecieron el efecto promotor

de tumores de la microcistina. Un fenómeno similar se había observado en el ácido ocadaico, una toxina procedente de algas marinas, que estimulaba el desarrollo de tumores al generar una excesiva fosforilación de las proteinas celulares (Cohen et al., 1990). Este ácido ocadaico actúa sobre las fosfatasas 1 y 2A, y los trabajos de Yoshizawa et al. (1990) mostraron que la microcistina era capaz de competir con él por los receptores en cuestión. La acción de las microcistinas fue confirmada por diversos grupos (Honkanen et al., 1990; Eriksson et al., 1990) y resultó ser bastante específica sobre fosfatasas 1 y 2A, no actuando sobre otras fosfatasas o quinasas, ni sobre fosfatasas de bacterias o cianobacterias (Mackintosh et al., 1990). También pudo establecerse más adelante que el aminoácido ADDA resultaba fundamental a la hora de expresar el efecto tóxico de las microcistinas, en tanto que es responsable de la inhibición de la acción enzimática al unirse al residuo de cisteína que estas fosfatasas poseen en su centro catalítico o al situarse en el bolsillo hidrofóbico del centro activo (Mackintosh et al., 1995; Runnegar et al., 1995; Goldberg et al., 1995). Modificaciones estructurales o la eliminación del ADDA se traducen en una reducción de la toxicidad (Abdel-rahman et al., 1993).

Estos resultados permiten establecer que la microcistina, mediante su aminoácido ADDA, es una toxina cuyos efectos principales pueden detectarse en eucariotas, y que actúa mediante una extraordinaria inhibición de dos fosfatasas esenciales. Esta inhibición da como resultado una variedad de efectos nocivos, algunos de los cuales se resumen a continuación.

Uno de los primeros efectos tóxicos observados fue que la inhibición de la actividad fosfatasa da lugar a la rápida deformación de hepatocitos aislados. Este fenómeno se explicaba con una hiperfosforilación del citoesqueleto, y concretamente de las citoqueratinas (Ohta et al., 1992). El hecho de que el daño provocado por microcistinas se centre en el sistema hepático se debe a que para poder actuar en el interior celular, la microcistina requiere de un transportador que le permita atravesar la membrana celular. El transportador de ácidos biliares reconoce a las microcistinas, y así permite su transporte hacia el interior de los hepatocitos.

Estudios más recientes sugieren que el daño provocado por microcistinas en hepatocitos no se restringe al citoplasma, sino que pueden ser capaces de penetrar en el núcleo celular, con lo que podrían afectar a las fosfatasas nucleares. El efecto de dicha

afección sería dramático en tanto podrían relacionarse con la muerte programada (apoptosis) de los hepatocitos. Esta apoptosis se puede activar mediante factores extrínsecos que actúan sobre receptores en la superficie de la célula o mediante señales provenientes del interior celular, debidos en muchas ocasiones a la presencia de compuestos tóxicos (Lockshin and Zakeri, 2001). El hecho de que haya sido posible prevenir el rápido estímulo de la apoptosis por parte de las microcistinas mediante la inhibición de enzimas quinasas indica que dicha muerte programada de las células se deba en origen a la hiperfosforilación de algunas estructuras. La vía mediante la cual esta hiperfosforilación se traduce en apoptosis es aún discutida, sugiriéndose vías como el estrés oxidativo o el daño al ADN. Finalmente mencionar que la apoptosis puede ser estimulada por microcistinas en cualquier célula a la que pueda acceder (Mankiewicz et al., 2001).

En cuanto a los posibles efectos teratogénicos, en mamíferos no se ha podido establecer aún una postura clara acerca de los posibles efectos de las microcistinas. En peces y anfibios, sí se ha demostrado que la exposición de embriones a extractos de cianobacterias o toxina pura se traduce en un incremento de malformaciones (Oberemm et al., 1997, 1999; Jacquet et al., 2004).

Ya desde muy pronto (Falconer et al., 1988) se sugirió, gracias a un estudio en ratones, la posibilidad de que una exposición crónica a dosis subletales de microcistina pudiera fomentar el cáncer de hígado. Dicha actividad se basaría en un esquema de carcinogénesis multifase. Es decir, que son varios los factores que secuencialmente contribuyen a la expresión del cáncer. Una mutación inicial podría, por ejemplo, no expresarse hasta recibir otros estímulos, propios o exógenos. En ratones, se ha comprobado dicha función de estímulo por parte de las microcistinas en el cáncer de piel, si bien asumiendo que posiblemente se deba a un proceso indirecto (Falconer y Buckley, 1989; Shen et al., 2003), en el cáncer de hígado (Nishiwaki-Matsushima et al., 1992) y en el cáncer de colon (Humpage et al., 2000).

Sin embargo, para evaluar correctamente la potencial capacidad promotora de tumores en el caso de las microcistinas, es necesario disponer de completos estudios epidemiológicos. Un estudio de esta índole se realizó en China (Yu, 1989, 1995). Se observaron áreas y pueblos con presencias muy elevadas de cáncer de hígado, contabilizándose hasta 60 casos por 100.000 habitantes. La identificación de los

posibles factores de riesgo dio como resultado la contaminación de maiz por aflatoxinas y la presencia de hepatitis B y C, pero destacaba también la correlación entre el mayor número de casos de cáncer hepático y el consumo de agua de acequias y estanques. La construcción de nuevos pozos en estas zonas resultó, al parecer, fundamental para disminuir la incidencia del cáncer de hígado hasta valores normales. Se indicó que el motivo por el cual el consumo de agua de acequias y estanques suponía un mayor riesgo de contraer cáncer hepático era la contaminación por microcistinas, si bien las concentraciones detectadas en estos sistemas eran bajas (Ueno et al., 1996). También en China, más recientemente se han realizado estudios epidemiológicos que correlacionan el cáncer de colon con el consumo de agua de ríos y estanques, y que incluso establecen una correlación positiva entre la concentración de microcistinas en el agua de consumo y la incidencia de dicho cáncer de colon (Zhou et al., 2002). Finalmente, estudios recientes en Serbia sugieren las microcistinas como uno de los factores riesgo clave para explicar la alta incidencia de cáncer de hígado en dicho país (Jukovic et al., 2008; Svircev et al., 2009).

Como se puede observar, el abanico de efectos nocivos protagonizados por las microcistinas es amplísimo, muchos de ellos debidos a exposiciones crónicas. Pero también las exposiciones agudas tienen consecuencias letales, tal y como se puede comprobar observando la dosis letal 50 (LD_{50}) de la microcistina LR y comparándola con otros agentes tóxicos que pueden encontrarse en aguas de consumo (tabla 1.3).

Compuesto	LD_{50} oral (mg/kg)
Antracina	850
Cobre	400
Acrilamida	100-270
Clorpirifos	60
Paratión	5
Microcistina LR	5
Cilindrospermopsina	6 (en 7 días)
Saxitoxina	0,12

Tabla 1.3. Toxicidad comparada de posibles contaminantes de aguas potables en roedor expresada como dosis letal 50 (dosis oral responsable de la mortandad del 50% de los indiciduos en 24 h) según Falconer (2005)

Finalmente, hemos de comentar que la toxicidad de las microcistinas varía enormemente de unas especies químicas a otras. Así, por ejemplo, la LD_{50} de la microcistina LR, en vía intraperitoneal, es doce veces menor que la de la microcistina RR (Sivonen y Jones, 1999). Esta diferencia de toxicidad se debe atribuir a otros factores que no sean la propia inhibición de la actividad fosfatasa, ya que las diferencias entre las constantes de inhibición de una y otra son muchísimo menores. Resultados experimentales en ratones sugieren que los procesos de detoxificación y excreción podrían ser responsables en buena medida de estas diferencias de toxicidad observadas (Ito et al., 2002)

1.2.1.4. Organismos productores

La tabla 1.2 mostraba el enorme número de especies capaces de producir microcistinas. En el área mediterránea, el género responsable en un mayor número de ocasiones de la producción de microcistinas es *Microcystis* (Cook et al., 2004, Carrasco et al., 2006). A su vez, dentro de este género, diversos trabajos han permitido establecer un ranking en cuanto al potencial de producción de microcistina de las distintas especies que componen este género. En este sentido, Via-Ordorika et al. (2004) sugieren que el orden de probabilidad sería *M. aeruginosa – M. flos-aquae – M. wesenbergii*. *M. aeruginosa* ha recibido además mucha atención por su facilidad de generar scums extraordinariamente tóxicos y por su amplísima distribución geográfica, desde ambientes fríos – si bien parece estar ausente de las zonas polares (Vincent, 2000) – hasta zonas tropicales. Desde lagos escandinavos en latitudes cercanas a los 60º N (Skulberg, 1996) hasta lagos neozelandeses cercanos a los 45º S (Walsby y McAllister, 1987), *M. aeruginosa* está presente en prácticamente cualquier clima.

Otro género habitualmente productor de microcistinas es *Planktothrix*, concretamente las especies *P. agardhii* y *P. rubescens*. En este caso, la probabilidad de producir microcistinas es elevadísma, especialmente en lo que afecta a *P. rubescens.* Por otra parte, la presencia de cepas de *Planktothrix* productoras de microcistina se ha centrado especialmente en el continente europeo (Fastner et al., 1999; Sivonen et al., 1990). Mencionar finalmente que *Planktothrix* tiene la peculiaridad de crecer en el metalimnion, aprovechando de esta forma los nutrientes procedentes del hipolimnion, de forma que puede originar importantísimos afloramientos en profundidad (ej.: Halvstedt et al., 2007)

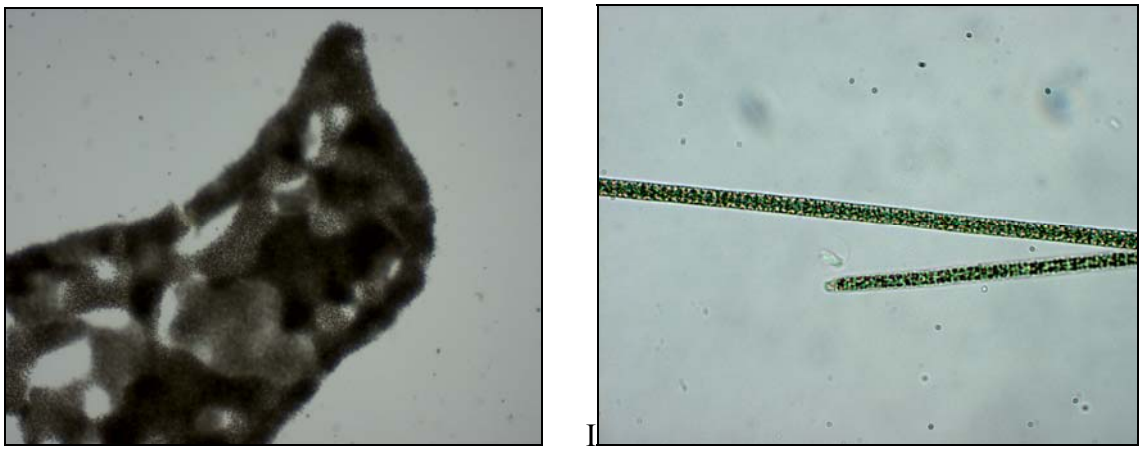

Foto 1.2. Microcystis aeruginosa (4x) y Planktothrix agardhii (40x) (fuente: Samuel Cirés)

1.2.1.5. Legislación

La predominancia de las microcistinas se ha traducido también en que han sido las primeras cianotoxinas para las que la Organización Mundial de la Salud (OMS) ha establecido un valor guía. Este valor guía indica que en aguas de consumo no debiera rebasarse una concentración de 1 μg l^{-1} (Chorus y Bartram, 1999). Dicha sugerencia ha sido incorporada de forma literal o ligeramente modificada en la legislación de diversos países, como Brasil, Canadá, Polonia, República Checa, Francia o España, mediante el RD 140/2003 (Chorus, 2005).

En aguas de recreo, aún se trabaja en la elaboración de valores guía. Aún así, algunos países han establecido normas o recomendaciones en este sentido. En el caso de los Países Bajos se establecen diversos niveles de alerta según la presencia de microcistinas. En la mayoría de los países sin embargo, el control de las zonas de baño se basa en factores como el número de células de cianobacterias, la inspección visual de la zona de baño o el estudio del estado trófico (Chorus, 2005). En España, en el RD 1341/2007, basado a su vez en la Directiva Europea 274/2006, a la hora de caracterizar las aguas de baño, se incluye el estudio de la propensión a la proliferación de cianobacterias y el control del riesgo de salud asociado.

1.2.2. Cilindrospermopsina

1.2.2.1. Estructura

La cilindrospermopsina, como ya se ha comentado, fue inicialmente aislada de un cultivo de *Cylindrospermopsis raciborskii*, a raiz del conocido como "Palm Island Mistery Disease", del cual se darán detalles a la hora de exponer el efecto tóxico de esta sustancia. Rápidamente fue posible establecer la estructura de este nuevo compuesto (Ohtani et al., 1992), la cual se muestra en la figura 1.7 y se compone de un derivado guanidino combinado con un grupo hidroximetiluracilo. Una peculiaridad de esta molécula es su doble carga (positiva y negativa), la cual la hace muy soluble en agua. A diferencia de la microcistina, no existe un elevado número de variantes de esta toxina. Sí se han descrito dos compuestos, la deoxycilindrospermopsina (Norris et al., 1999) y la 7-epicilindrospermopsina (Banker et al., 2000) las cuales se diferencia respectivamente por la ausencia de un oxígeno y por la posición del grupo –OH en el carbono 7. En todo caso, la variación estructural entre cilindrospermopsina y 7-epicilindrospermopsina no parece afectar en grado alguno a su toxicidad.

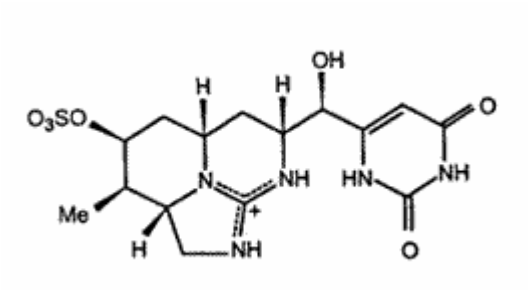

Figura 1.7. Estructura química de la cilindrospermopsina (fuente: cyanobacteria-platform.com)

1.2.2.2. Síntesis y liberación

También en el caso de la cilindrospermopsina, el estudio de sus rutas de síntesis se ha realizado mediante el uso de posibles precursores marcados radioactivamente, y mediante la búsqueda de regiones genéticas que pudieran estar involucradas en la síntesis de este compuesto.

En cuanto a la primera vía (Burgoyne et al. 2000), se pudo identificar la glicina, cinco grupos acetato y un grupo S-adenosilmetionina como precursores, siendo la formación de acetato guanidino – a partir de la glicina – el primer paso de la síntesis de la cilindrospermopsina. A continuación se construiría la estructura carbónica de la molécula mediante la adición de grupos acetato. A este esqueleto se le sumarían reacciones de metilación, sulfatación, ciclación o keto reducción para completar la biosíntesis de la molécula. En todo caso, esta vía dejaba aún algunas incógnitas por despejar, como serían los orígenes del grupo guanidino o uracilo.

La segunda vía fue iniciada – para la cilindrospermopsina – por Schembri et al. (2001), que identificaron dos posibles regiones involucradas en la síntesis de la cilindrospermopsina en *Cylindrospermopsis raciborskii* y *Anabaena bergii*. Esta identificación se basaba en la asunción de que la producción de la toxina estaría ligada a la actividad de péptido y poliquétido sintetasas. Los autores fueron capaces de demostrar que la presencia de estos genes se correlacionaba directamente con la capacidad de producir cilindrospermopsina en distintas cepas. Posteriormente, Shalev-Alon et al. (2002) pudieron secuenciar este cluster en *Aphanizomenon ovalisporum*, identificando tres genes *aoaA*, *aoaB* y *aoaC* responsables de parte de la biosíntesis del compuesto. *aoaA* codificaría una amidinotransferasa (AoaA) que catalizaría la síntesis del acetato de guanidino, el primer paso de la biosíntesis, tal y como hemos descrito con anterioridad. Esta amidinotransferasa guarda gran homología con amidinotransferasas arginina:glicina en vertebrados. Esto, junto a que el estudio de su funcionalidad revelaba aparentemente similitud con amidinotransferasas encargadas de arginina y glicinas en humanos (Kellmann et al., 2006) sugiere que el compuesto desconocido que Burgoyne et al. (2000) predecían como precursor del acetato de guanidino podría ser efectivamente la arginina. A continuación, el acetato de guanidino sería reclutado por AoaB, un híbrido de péptido sintetasa no ribosomal y poliquétido sintetasa, el cual lo reconoce por el dominio de adenilación. Junto con AoaC, una poliquétido sintetasa, serían responsables de la ampliación de la cadena poliquetídica. Estos tres genes han sido detectados exclusivamente en cepas productoras de cilindrospermopsina en *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Umezakia natans* y *Aphanizomenon flos-aquae* (Schembri et al., 2001; Shalev-Alon et al., 2002; Kellmann et al., 2006; Preussel et al., 2006).

Recientemente, Mihali et al. (2008) han publicado la descripción completa de la ruta de síntesis de la cilindrospermopsina, completando con ello los trabajos hasta aquí descritos (figura 1.8). Estos trabajos confirman los pasos iniciales sugeridos, siendo CyrA, CyrB y CyrC homólogos de AoaA, AoaB y AoaC respectivamente. Sin embargo, difieren en la formación de los anillos de la cilindrospermopsina, los cuales atribuyen a ataques de grupos guanidinos sobre dobles enlaces entre carbonos, reacciones que son coherentes con las condiciones para la formación de anillos según Baldwin et al. (1977). De esta manera, la ciclación no tendría que estar catalizada, sino que se daría de forma espontánea a lo largo de la ruta de síntesis.

A los tres pasos iniciales les sigue CyrD y CyrE, también poliquétido sintetasas, responsables cada una de la adición de un nuevo grupo acetato y otros procesos como la reducción de un grupo keto o la creación de dobles enlaces entre C-9 y C-10 y C-7 y C-8 respectivamente. La destrucción de estos dobles enlaces mediante los nitrógenos en N-19 y N-18 llevará a la formación del segundo y tercer anillo. CyrF, última poliquétido sintetasa involucrada, añade un nuevo grupo acetato. CyrG y CyrH son responsables de la formación del grupo uracilo. Mihali et al. (2008) atribuyen esta formación a la transferencia de un nuevo grupo guanidino – procedente de por ejemplo arginina o urea – a los carbonos en posición 4 y 6. CyrI, CyrJ y CyrN son candidatos a participar en los procesos que añadirán un grupo sulfato en C-12 y un grupo hidroxilo en C-7.

Finalmente, resulta muy interesante el descubrimiento de CyrK, un producto similar a proteinas responsables de exportar compuestos tóxicos de la familia NorM, reguladas mediante Na^+. Basándose en esta similitud, los autores sugieren que CyrK sea responsable de la excreción de cilindrospermopsina al medio.

Tal descubrimiento, de confirmarse, sería de gran interés al contribuir a explicar la gran cantidad de cilindrospermopsina extracelular que se ha observado en cultivos de laboratorio. Esta importante presencia de la fracción extracelular es consecuente con la idea general de que las toxinas alcaloides son más fácilmente liberadas que las peptídicas. En cultivos batch de *Cylindrospermopsis raciborskii*, Saker and Griffiths (2000) observaron que cerca del 20% de la toxina puede ser excretada durante la fase exponencial, y hasta un 50% en fase estacionaria.

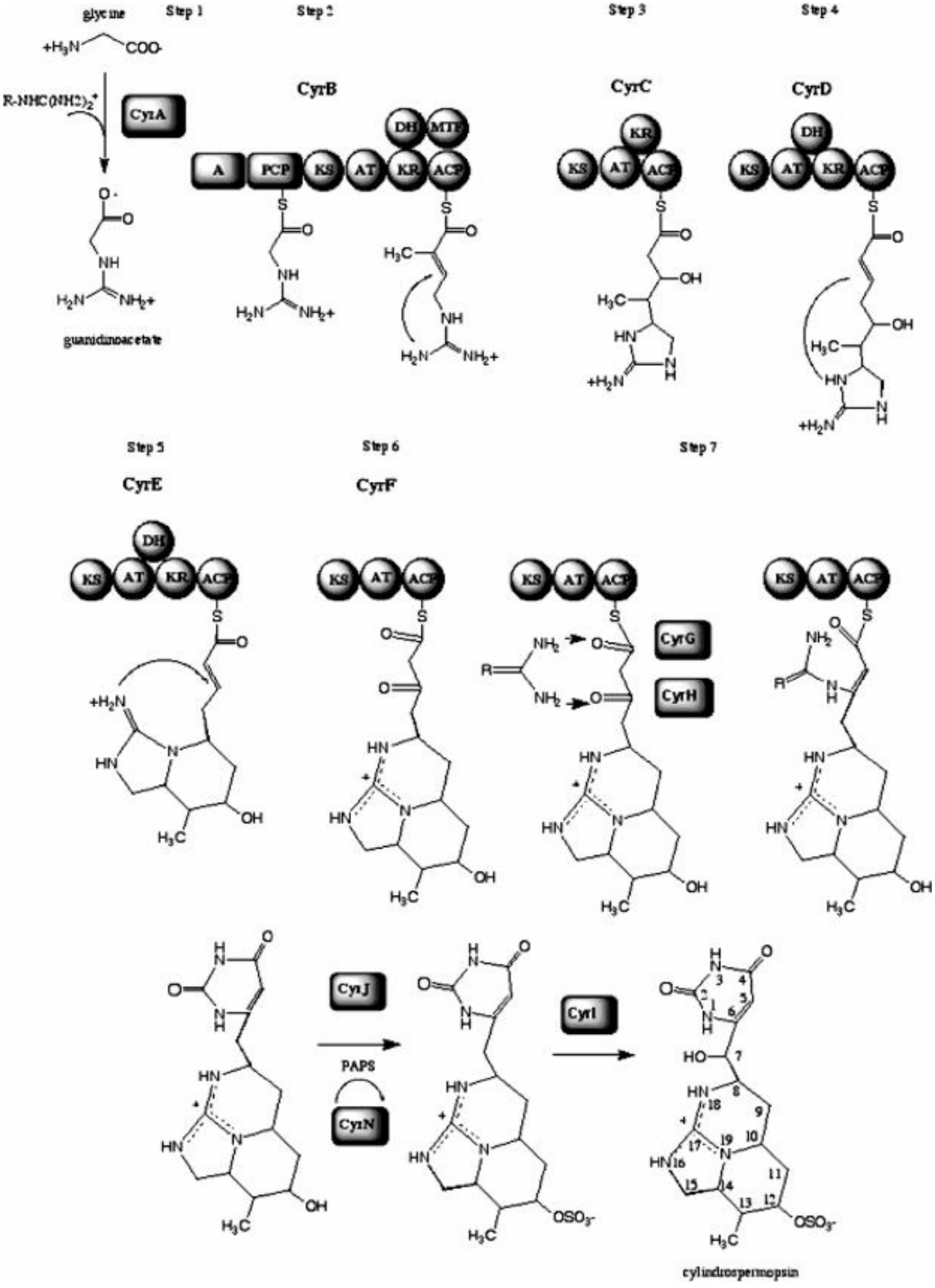

Figura 1.8. Ruta biosintética de la cilindrospermopsina (Mihali et al., 2008)

En trabajos realizados en nuestro laboratorio (Timón, 2007) con *Aphanizomenon ovalisporum* en cultivos batch también se observa que la importancia de las fracciones extra- e intracelular se va igualando según avanza el crecimiento del cultivo, llegando

finalmente la fracción extracelular a valores de entre el 40 y 50%. Además se observó que una menor irradiancia recibida puede aumentar la producción global de toxina, al tiempo que a muy bajas irradiancias (15 μEinstein m^{-2} s^{-1}) la presencia de la misma en fracción disuelta decae. Preussel et al. (2008) también dedujeron que debía existir liberación activa de cilindrospermopsina en sus estudios con *Aphanizomenon flos-aquae* en cultivos semicontinuos. Altas irradiancias con bajas temperaturas dieron lugar a las tasas de cilindrospermopsina extracelular más elevadas, llegando a suponer un 58% de la toxina total.

En datos de campo, que serán mostrados en mayor detalle en el capítulo tercero se ha observado, por ejemplo, que en blooms de *Cylindrospermopsis raciborskii* la importancia de la fracción extracelular podía suponer entre un 19 y un 98% de la toxina total (Chiswell et al., 1999). Shaw et al (1999) cuantifican la importancia de la fracción extracelular en *Aphanizomenon ovalisporum* como mínimo entorno al 85%. También, como en el caso de la microcistina, se ha descrito la estabilidad de la toxina frente a temperatura y pH (Chiswell et al., 1999)

1.2.2.3. Efecto tóxico

Mientras que en las microcistinas el efecto tóxico se centra en el sistema hepático por la necesidad de transportadores, la cilindrospermopsina parece actuar sobre un mayor rango de órganos, al poder penetrar en células expuestas con independencia de la disponibilidad de dichos transportadores (Chong, 2002). Una vez en el interior celular, se ha comprobado el efecto de inhibición de síntesis de proteínas. Dicha comprobación pudo ser realizada in vitro (Terao et al., 1994), y explica algunas de las alteraciones observadas en células expuestas, como la disociación de ribosomas del retículo endoplasmático rugoso. Posteriormente, se pudo evidenciar que la toxina actuaba como un inhibidor de los procesos de elongación en la síntesis de proteinas. Además, Froscio et al. (2003) describieron que los procesos responsables de esta inhibición eran irreversibles. Sin embargo, esta inhibición de la síntesis de proteínas parece estar desacoplada de la muerte celular observada en hepatocitos, en cuanto al tiempo y a la concentración requerida. En este sentido, es significativo que la LD_{50} a las 24 horas es significativamente mayor que a los 7 días (Hawkins et al., 1997).

La explicación dada para este fenómeno reside en que la cilindrospermopsina pura es responsable de la inhibición de la síntesis de proteínas, provocando una intoxicación más lenta, mientras que un metabolito de la cilindrospermopsina parece ser responsable de un efecto tóxico más agudo. En este sentido, la adición de inhibidores del citocromo P450 protege a las células expuestas del efecto tóxico más agudo (Runnegar et al., 1995; Froscio et al., 2003). En animales a los que se les suministra esta posible protección los resultados han sido ambiguos (Norris et al., 2002). El hecho de que los distintos órganos alberguen distintas actividades del citocromo P450 explicaría la distinta sensibilidad de las células de estos órganos a la cilindrospermopsina.

La presencia de un grupo uracilo en la estructura de la cilindrospermopsina hace presumir cierta facilidad para interaccionar con los grupos adenina de ARN y ADN. De hecho, Falconer (2005) sugiere que la construcción de modelos de interacción entre cilindrospermopsina y ADN muestran la posibilidad de que la toxina se intercale entre los nucleótidos de la doble hélice, generando un potencial efecto mutagénico, clastogénico o carcinogénico. De hecho, Humpage et al. (2000) observaron dicho daño en células linfáticas humanas, al describir el incremento de micronúcleos formados con adición de cilindrospermopsina. Shen et al. (2002) observaron el efecto directo de la cilindrospermopsina sobre la integridad de ADN extraído, reduciéndose la longitud mediana de dicho ADN entorno a un 25%.

Además de los estudios que sugieren este potencial carácter carcinogénico de la cilindrospermopsina, se ha de mencionar un muy interesante estudio de 30 semanas en el cual esta toxina fue administrada oralmente a ratones en diferente número de dosis (Falconer y Humpage, 2001). Tras el periodo de experimentación, de los 53 ratones tratados con cilindrospermopsina, 5 habían desarrollado tumores. En los casos control, sin adición de toxina, no se detectaron tumores.

1.2.2.4. Organismos productores

La cilindrospermopsina – y sus productores – han sido descritos principal e inicialmente en Oceanía, en buena medida a partir del ya mencionado Palm Island Mistery Disease. En estos casos, *Cylindrospermopsis raciborskii*, del que la toxina toma el nombre, era identificado como organismo productor. Desde entonces, la toxina ha mantenido un elevado nivel de alarma, al aparecer recurrentemente en los sistemas de suministro de agua (ej.: Bourke et al., 1983, Shaw et al., 1999). La toxina cobró importancia también en Israel, donde fue detectada en la principal fuente de agua dulce del país, el lago Kinneret (Banker et al., 1997). En este caso, el organismo productor era *Aphanizomenon ovalisporum*, especie que poco después se sumó a *Cylindrospermopsis raciborskii* como responsable de la presencia de esta toxina en Australia (Shaw et al., 1999). Estas dos especies son todavía consideradas las principales productoras de cilindrospermopsina, si bien resulta interesante destacar la identificación de *Aphanizomenon flos-aquae* como potencial productor de esta toxina (Preussel et al., 2006), en tanto que se trata de una de las especies más frecuentes en aguas españolas, acompañando en muchos casos al género *Microcystis* (Carrasco, 2007). Por otra parte, tal y como se muestra en la tabla 1.2, con gran frecuencia se suman nuevas especies al listado de potenciales productores de cilindrospermopsina.

Foto 1.3. Cylindrospermopsis raciborskii (40x) y Aphanizomenon ovalisporum (40x) (fuente: Samuel Cirés)

También es llamativo que muchas de estas especies han sido identificadas recientemente en climas más fríos. Y es que la cilindrospermopsina resulta especialmente interesante por la paulatina expansión geográfica de algunos productores hacia latitudes menos calurosas (Briand et al., 2004). Así, los comienzos del siglo XXI

han sido testigos de una repentina y masiva descripción de episodios tóxicos y de la identificación de nuevos productores en aguas europeas. Inicialmente detectada sólo en bajas concentraciones (Fastner et al., 2003), ya en 2004, se detectó el primer bloom de cianobacterias productoras de cilindrospermopsina en aguas europeas, concretamente en el embalse de Arcos, Cádiz (Quesada et al., 2006). A estos episodios les han seguido otros muchos a lo largo de todo el continente (Bogialli et al., 2006; Rücker et al., 2007; Bláhová et al., 2009; Kokocinski et al., 2009). Es llamativo que en ninguno de estos casos observados en el continente europeo se haya podido describir la síntesis de cilindrospermopsina por *Cylindrospermopsis raciborskii* – considerado principal productor en prácticamente el resto del mundo (Bernard et al., 2003; Neilan et al., 2003; Chonudomkul et al., 2004; Berger et al., 2006). En relación a esto Haande et al. (2008) describieron las diferencias genéticas entre cepas de *Cylindrospermopsis raciborskii* de diversas regiones geográficas, contribuyendo con ello a una posible explicación del distinto comportamiento tóxico de las cepas

1.2.2.5. Legislación

En el caso de la cilindrospermopsina, diversos trabajos han sido realizados de cara a la obtención de un valor guía, proponiendo Humpage y Falconer (2003) una concentración de 1 $\mu g\ l^{-1}$. Sin embargo, y posiblemente dada la limitada distribución geográfica de la toxina en el pasado, únicamente algunos países, como Brasil o Nueva Zelanda han incorporado estos valores a su legislación, mientras que en Australia existen valores recomendados (Chorus, 2005)

1.3. Episodios de intoxicaciones atribuibles a cianobacterias tóxicas

Los afloramientos tóxicos de cianobacterias no son un fenómeno reciente. De hecho, la primera narración de un bloom tóxico puede datarse en 1878 (Francis, 1878). George Francis había sido contratado por el Gobierno de Australia del Sur para estudiar el envenenamiento de animales en las orillas del lago Alexandrina. Las observaciones de Francis se centraron en el bajo nivel del agua en el lago, en lo caliente de la misma y en la presencia masiva de fitoplancton flotando en la superficie del lago: "*where it was forming a thick scum like green oil paint, some two to six inches thick, and as thick and pasty as porridge*". Además de esto, destacaba la presencia de un intenso pigmento azulado. Francis identificó la cianobacteria tóxica como *Nodularia spumigena*, y

observó que, una vez ingerida una cantidad suficiente del agua afectada, los animales morían en pocas horas.

La evidencia de efectos dañinos atribuibles a cianotoxinas se nutre de tres fuentes. Por un lado, disponemos de estudios toxicológicos – como los ya descritos para microcistinas y cilindrospermopsina – que demuestran el potencial nocivo de estas sustancias. Por otra parte, podemos disponer de estudios epidemiológicos de poblaciones humanas que muestran síntomas compatibles con intoxicaciones por cianotoxinas. Finalmente existe un gran número de casos de intoxicaciones en animales que – si bien no son extrapolables al ser humano – ofrecen casos reales de efectos bajo condiciones naturales. En este apartado nos centraremos en reunir algunos de los más significativos casos de intoxicaciones humanas y animales atribuidas a cianotoxinas.

1.3.1. Intoxicación de poblaciones humanas por aguas de consumo

En el apartado 1.2.2 describimos el posible efecto carcinogénico de exposiciones crónicas a microcistinas en aguas de bebida. Sin embargo, también existen algunos casos significativos de intoxicación por exposiciones agudas. Quizás el más significativo sea el de las proximidades del embalse de Itaparica en Brasil. Durante el año 1988 se registraron cerca de 2000 casos de gastroenteritis durante 42 días, 88 de estos casos resultaron fatales (Teixieira et al., 1993). Tras el minucioso estudio de posibles orígenes bacteriológicos, viriológicos y toxicológicos, se demostró que el desencadenante de la epidemia eran las toxinas producidas por las cianobacterias presentes en el embalse. La comunidad de cianobacterias estaba dominada por *Anabaena* y *Microcystis*.

Otro caso destacado es el ocurrido en el embalse de Malpas (Nueva Gales del Sur, Australia), habitualmente afectado por blooms de *Microcystis aeruginosa*, en los cuales se identificó la presencia de microcistina YM (Botes et al., 1984). En este embalse durante el año 1981 se decidió combatir un bloom especialmente denso de *Microcystis aeruginosa* mediante el uso de alguicidas, concretamente sulfato de cobre (Falconer et al., 1983), lo cual habría permitido el paso de las toxinas a la fracción extracelular. El agua así tratada fue consumida por los habitantes de la cercana ciudad de Armidale. Estudios realizados sobre muestras obtenidas antes, durante y después del bloom demostraron severos daños en el sistema hepático de los habitantes que recibían

el agua de consumo desde el embalse de Malpas, no así en otros vecinos. Esto sugiere que la presencia y posterior lisis del bloom de *Microcystis* eran responsables del daño hepático observado (Falconer et al., 1983).

Otro caso de sumo interés es el denominado Palm Island Mistery Disease de 1979 (Byth, 1980). Como su propio nombre indica, la detección de súbitas y agudas dolencias detectadas en 138 niños y 10 adultos en la isla tropical de Palm Island (Queensland, Australia) no pudo ser fácilmente explicada. Las dolencias descritas incluían hígado hinchado, dolor abdominal, vómitos, daño renal o fuertes diarreas con presencia de sangre en heces. Ochenta y cinco pacientes tuvieron que ser trasladados a hospitales fuera de la isla debido a la gravedad de los síntomas, algunos a unidades de cuidados intensivos. Posteriores estudios identificaron como causa más probable de estos sucesos la liberación de cianotoxinas por la aplicación sulfato de cobre en el embalse de Solomon (Bourke et al., 1983). El posterior estudio de dicho embalse mostró que eran habituales los blooms de *Cylindrospermopsis raciborskii* y *Anabaena circinalis*. La cepa de *C. raciborskii* resultó extremadamente tóxica. Administrada a ratones se observaron daños severos en el sistema hepático, pero también en riñón, intestino delgado, pulmón y glándulas adrenales, estableciéndose la muy probable responsabilidad de *C. raciborskii* en los casos observados en Palm Island.

Existen otros muchos episodios que pueden ser atribuidos a cianobacterias tóxicas, como el masivo brote de entre 4000 y 7000 casos de dolencias abdominales, vómitos y diarreas en Charleston (Virginia Occidental, EE.UU) en 1931 (Tisdale, 1931) o el incremento de casos de gastroenteritis en Harare (Zimbabwe) coincidente con el bloom anual de *Microcystis aeruginosa* (Zilberg, 1966).

1.3.2. Intoxicación de poblaciones humanas en aguas de recreo

Las posibles intoxicaciones en aguas de recreo son incluso más difíciles de detectar, en tanto que no existe un registro claro de posibles personas afectadas. Aún así, cuando este círculo de posibles afectados es limitado, surgen casos de sumo interés. A modo de ejemplo, en 1989 un grupo de 20 soldados ingleses realizaba ejercicios en el embalse de Rudyard, un sistema que puede recibir entorno a 100.000 visitantes anualmente. Concluidos los ejercicios en el embalse, hasta 10 reclutas presentaron gastroenteritis, garganta irritada, ampollas en la boca, dolor abdominal, fiebre,

consolidación pulmonar y vómitos. En el embalse se detectó la presencia de *Microcystis aeruginosa* y microcistina LR (Turner et al., 1990).

Recientemente, un forense en Wisconsin (EE.UU) concluyó que la muerte de un adolescente se podía deber a que había estado buceando en un estanque en el cual se había desarrollado un masivo scum de *Anabaena flos-aquae* productor de anatoxina-a, si bien la asignación de autoría de esta muerte a anatoxinas no puede considerarse científicamente probada. Tras la exposición el joven y un amigo sufrieron vómitos, diarrea y dolor abdominal, pereciendo el joven tras sólo 48 horas (Behm, 2003; Falconer, 2005).

En cuanto a aguas de recreo, ha de mencionarse los estudios epidemiológicos de Pilotto (1997, 2004), en los cuales se pudo establecer una relación entre diversos síntomas como diarreas, vómitos, úlceras bucales, irritaciones y fiebre con la presencia y la densidad de cianobacterias. Finalmente, hemos de destacar que un estudio realizado en ratones indica que la toxicidad de microcistina LR es un orden de magnitud mayor cuando la ingesta es por por inhalación de aerosoles que cuando es por vía oral (Benson et al, 2005). Este es un aspecto muy a tener en cuenta en aguas de recreo, dada la cantidad de actividades recreativas que pueden dar lugar a gran cantidad de aerosoles.

1.3.3. Intoxicación de poblaciones humanas por otras vías

Si bien las dos vías previamente descritas pueden ser las más evidentes, existen otras posibles rutas por las cuales las cianotoxinas pueden alcanzar a poblaciones humanas. En este sentido, el ejemplo más trágicamente conocido es el uso de agua contaminada por microcistinas en una clínica de diálisis en Brasil. En 1996, en una clínica de diálisis en Caruaru, 116 pacientes – de un total de 131 tratados – se vieron afectados por náuseas y vómitos, debilidad muscular, dolor de cabeza, alteración de la capacidad visual y dolores gástricos. Cien de estos pacientes desarrollaron fallo hepático, con consecuencias fatales en 76 de estos casos. Al parecer, el mantenimiento de los sistemas de tratamiento del agua en la unidad de diálisis no había sido el adecuado, y este descuido había provocado la intoxicación. De hecho, posteriormente se estudiaron los filtros de dichos sistemas de tratamiento, detectándose hasta 2,2 μg/g de microcistina y 19,7 μg/g de cilindrospermopsina. En el hígado de los pacientes afectados se detectaron desde 50 a 471 ng/g de microcistina (Carmichael et al., 2001).

Otras posibles vías, para las que sin embargo no existen aún casos de intoxicación descritos, serían por ejemplo la irrigación de cultivos mediante agua afectada por cianotoxinas o el uso de cianobacterias potencialmente tóxicas en suplementos dietéticos. Saker et al. (2005), por ejemplo, detectaron microcistina y genes de microcistina sintetasas en suplementos alimenticios que contenían *Aphanizomenon flos-aquae*.

1.3.4. Intoxicaciones animales

La mayoría de los casos de intoxicaciones animales se debe a que dichos animales hubieran bebido de pequeños lagos o embalses afectados por afloramientos cianobacterianos. Sin embargo existen también casos de intoxicaciones masivas. Por ejemplo, ya entre 1943 y 1945, Steyn describía mortandades masivas de ovejas y reses en Sudáfrica (ej.: Steyn, 1945). De hecho, en estos trabajos se constataba que miles de cabezas de ganado habían muerto intoxicadas en los 25 o 30 años anteriores. Aún hoy siguen produciéndose fenómenos de muerte masivas en Sudáfrica (Harding and Paxton, 2001)

En Australia, durante el año 1991, se detectó un extensísimo bloom de *Anabaena circinalis*, que llegó a externderse a lo largo de 1000 km en el río Darling. Se constató la muerte de aproximadamente 2000 cabezas de ganado vacuno y bovino (Falconer, 2005). Finalmente, por mencionar un caso en territorio español, en el Parque Nacional de Doñana, se observó la muerte masiva de flamencos atribuida a microcistinas (López-Rodas et al., 2008).

Si bien hasta aquí se han podido mencionar sólo algunos de los casos más llamativos de blooms tóxicos, episodios similares se han ido repitiendo en todo el mundo, convirtiendo el fenómeno de los afloramientos de cianobacterias tóxicas en un problema global.

2. Hipótesis y Objetivos

El trabajo experimental desarrollado ha centrado su atención en las cianotoxinas microcistina y cilindrospermopsina. La elección de estas toxinas, estables químicamente y con una probada toxicidad, se ha debido a la predominancia de la primera en aguas españolas, al reciente descubrimiento y a la rápida expansión de la segunda en ambientes templados y a la aparentemente muy escasa presencia de otras toxinas – como las neurotoxinas – en aguas continentales españolas. Más allá de esto, microcistina y cilindrospermopsina sirven de excelentes representantes de los dos grupos más importantes de compuestos bioactivos en cianobacterias: polipéptidos y alcaloides de pequeño tamaño.

Probado el efecto nocivo de estas toxinas en humanos y animales, probablemente uno de los aspectos fundamentales a tratar es la correcta evaluación de las posibles vías de exposición. Nuestro trabajo entronca con dicha demanda al centrarse en el estudio de la dinámica de las toxinas una vez producidas por el organismo. Nuestro objetivo es lograr una profunda comprensión de los procesos dominantes que definen el destino de microcistinas y cilindrospermopsina en ambientes naturales. Esto permitirá mostrar la degradación, el secuestro o la disponibilidad por diversas vías de las cianotoxinas estudiadas. Una vez más, al trabajar con compuestos químicamente muy distintos, esperamos ser capaces de mostrar la diversidad de comportamientos que protagonizan cada una de ellas.

Nuestra hipótesis de partida plantea dos escenarios bien diferenciados para cada toxina. Las microcistinas se han detectado principalmente en el interior celular, por lo que esperamos que su destino en los sistemas naturales se vea ligado al de los organismos productores. Considerando la importancia de los procesos de sedimentación en el ciclo anual de muchos de estos potenciales productores, en principio otorgamos a la sedimentación otoñal un papel fundamental en la distribución de la microcistina en aguas naturales. Por contra, el alcaloide cilindrospermopsina es liberado en elevadas tasas, por lo que hipotetizamos que su destino en los sistemas naturales será independiente del organismo productor. De hecho, postulamos que los procesos de degradación *in situ* de la toxina extracelular serán los agentes que influirán en mayor medida en su distribución.

Para lograr la evaluación crítica de estas hipótesis, se ha recurrido a una serie de trabajos experimentales en campo y laboratorio. El extenso trabajo de campo muestra además la peculiaridad de realizarse en embalses bastante profundos, unos sistemas poco estudiados hasta la fecha en relación a las cianotoxinas. En concreto, los objetivos que nos hemos marcado para, en su conjunto, completar el entendimiento de las dinámicas de distribución y degradación de microcistinas y cilindrospermopsina son los siguientes:

En primer lugar (capítulo 3), se trató de obtener una visión global del fenómeno de las cianobacterias y las cianotoxinas en aguas españolas, basándose para ello en diversas campañas de muestreo en siete cuencas hidrográficas. Las muestras recogidas debían indicarnos las variaciones temporales y geográficas de las cianobacterias y, a su vez, servir para evaluar la importancia de microcistinas, cilindrospermopsina y anatoxina-a. En el caso de las dos primeras, prestamos especial atención al reparto de las mismas en fracción intra- y extracelular, en tanto que se esperaba encontrar grandes diferencias entre ambas toxinas y estas diferencias debían ser explicadas con los datos experimentales obtenidos más adelante.

La evaluación de la importancia de cilindrospermopsina en fracción extracelular hacía imprescindible la puesta a punto de un método eficaz de concentración de dicha toxina. El método a desarrollar debía ser efectivo tanto para la concentración de pequeñas concentraciones de toxina en muestras de campo complejas, como para la extracción de grandes cantidades de toxina desde medios de cultivo (capítulo 4).

Una vez liberadas al medio, las cianotoxinas se verán expuestas a factores de degradación de los cuales permanecían protegidos en el interior celular. Nuestro objetivo es testar la efectividad de dichos agentes degradadores *in situ*, pues será fundamental para la rápida reducción del riesgo asociado a cianotoxinas, especialmente en aquellas liberadas en mayor medida al medio extracelular. En el capítulo quinto hemos abordado la degradación mediada por actividad bacteriana, así como aquella provocada por la irradiancia solar.

En el caso de las microcistinas, liberadas en principio sólo con la muerte celular, sugerimos que la sedimentación de toxina asociada a organismos o restos celulares puede suponer un muy importante destino de esta toxina. Además, hipotetizamos que

estas toxinas pueden ser adsorbidas por distintos componentes orgánicos o inorgánicos del sedimento. De darse una sorción fuerte de las toxinas, esto equivaldría a un efectivo secuestro de la toxina, reduciendo drásticamente su disponibilidad. El capítulo sexto recoge los resultados relativos a ambos aspectos, así como los datos obtenidos a la hora de evaluar diversas técnicas para la extracción de cianotoxinas del sedimento.

2. Hypotheses & Aims

Our experimental work concentrated on the cyanobacterial toxins microcystin and cylindrospermopsin, chemically stable compounds of proven toxicity. Among the cyanobacterial toxins, microcystins seem to be most widely distributed, and also have been considered dominant in Spanish freshwaters. Cylindrospermopsins seem very interesting because of their novelty to European waters, and their apparently rapid invasion of milder latitudes. Further, these two toxins are representative for the two major groups of cyanobacterial toxins: polipeptides and small alcaloids.

Considering harmful effects of cyanobacterial toxins, possible exposure routes seem to be an aspect of greatest importance. We consider that a correct and complete evaluation of this exposure will only be achieved when understanding the fate of the toxin in the environment. Therefore, we tried to identify and characterize the dominating processes microcystins and cylindrospermopsin are subjected to, once they have been synthesized. Only then, sequestration, degradation or availabilty of the toxin in the environment may be predicted. Again, by choosing representatives of two very different groups of cyanobacterial toxins, we hope to detect a variety of behaviours to be expected at the field.

Our initial hypothesis predicts two very different scenarios for the studied toxins. On the one hand, microcystins have been detected mainly in the intracellular phase, therefore we expect their fate to be closely linked to the fate of the producing organisms. Considering the importance of sedimentation in the annual cycle of many toxic cyanobacteria, we suggest sedimentation, and especially autumnal sedimentation, to play a very important role in the distribution of microcystins in natural waters.

On the other hand Cylindrospermopsin, seems to be liberated in great amounts, this fact leading towards suggesting that its fate will be less linked to the producing

organisms. Instead, we expect in situ degradation of extracellular toxin to be the main agent in defining its distribution.

Both laboratory and field work has been accomplished in order to achieve a critical evaluation of these hypotheses. Considering field work, it is interesting to point out that it has been mainly carried out in deep, thermally stratified reservoirs, which so far have only received limited attention concerning the study of cyanobacterial toxins. The concrete objectives we have proposed to ourselves in order to fulfill the understanding of distribution and degradation dynamics of microcystins and cylindrospermopsin are as follows:

First of all (chapter 3), we tried to obtain a global vision of occurrence of cyanobacteria and cyanotoxins in Spanish freshwaters, based on samples obtained from seven basins. We were interested in understanding geographical and temporal variations and in evaluating the importance of microcystins, cylindrospermopsin and anatoxin-a. In the case of microcystins and cylindrospermopsin, special attention was paid to the naturally occurring distribution of intra- and extracellular toxin, as great differences between the two toxins were expected, and such differences had to be explained by the experimental data obtained.

The study of extracellular cylindrospermopsin and its behaviour in the environment firstly demanded an effective and reliable method which allowed to recover the toxin from the aqueous phase. Our aim was to develop a method useful both for the concentration of small amounts of toxin from environmental samples or experimental enclosures and for the recovery of high amounts of toxin from growth media (chapter 4).

Outside the producing organism, cyanobacterial toxins will be exposed to a variety of degradation agents, from which they were protected inside the cell. A rapid in situ degradation will be crucial in order to reduce the risks of exposure to the toxins, especially when considering toxins excreted to the environment in high amounts. Our aim was to test the efficiency of some of these agents under natural conditions. Chapter 5 will resume our results concerning bio- and photodegradation of microcystins and cylindrospermopsin.

As said before, the fate of microcystins is expected to be linked to the producing organism. Therefore, we suggest that sedimentation of microcystin inside settling organisms or attached to cell debris might be of greatest importance. Further, we believe that strong sorption of cyanobacterial toxins to organic or inorganic components of the sediments might be occurring, drastically reducing the availability of these compounds. Chapter six shows the results which allow the evaluation of both hypotheses, and also contains some data concerning extraction of cyanobacterial toxins from sediment samples.

3. Planktonic cyanobacteria and cyanobacterial toxins in Spanish freshwaters

Cyanobacterial blooms and the cyanobacterial toxins associated have become a worlwide phenomenon, possible due to the impact of human activities on water quality, but also because of the increasing interest in this issue and the improvement of analytical methods. During the development of this PhD thesis we have been constantly involved in the analysis of cyanobacteria and their toxins in reservoirs, which were sampled by our own or by diverse water authorities (CEDEX, Confederación Hidrográfica del Norte, Confederación Hidrográfica del Ebro, Ministerio de Medio Ambiente, Rural y Marino). These samplings were carried out in 63 waterbodies of seven different watersheds along six consecutive years. Although a greater body of data concerning some of these samplings is available in different publications, here we only present results obtained in our laboratoy. These results allow us to show a global picture of the occurrence of these organisms and their spatial and temporal distribution in Spanish feshwaters.

Besides the presence of cyanobacteria, our studies were focused on the production of microcystins, cylindrospermopsin and anatoxin-a by these organisms. We were interested in evaluating both the frequency and the magnitude of toxic episodes, and in the case of microcystins and cylindrospermopsin paid special attention to the distribution of the toxins in the intra-and extracellular phase.

3.1. Planktonic cyanobacteria and cyanobacterial toxins in Spanish freshwaters

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3.1.1. Abstract

This study describes the extended occurrence of both cyanobacteria and cyanobacterial toxins in 63 Spanish freshwaters, sampled in summer and autumn from 2003 to 2009. Cyanobacterial chlorophyll *a* was evaluated by fluorometry, and microcystins, cylindropsermopsin and anatoxin-a analysed by HPLC-PDA or LC-MS/MS. On a regional scale, cyanobacteria were widely distributed and especially dominated the sampled waterbodies from the Guadiana catchment area. On a temporal scale, August, September and October were identified as the months in which cyanobacteria achieved highest biomass, major growth takes place earlier in the southern watersheds. Microcystins are confirmed as a great matter of concern, as 50% of the studied waterbodies were affected by this toxin. Still, in more than half of these cases, maximum microcystin concentration was below 1 μg l^{-1}. While anatoxin-a was almost absent from the analysed samples, cylindrospermopsin, a toxin only recently found in Europe, was detected in 10% of the tested reservoirs in high concentration. This fact, linked to the high presence of the toxin in the extracellular phase, should be seriously considered.

3.1.2. Introduction

Surface waters are used in many parts of the world for drinking water supply or recreational use. In last decades, concern about the increased presence of harmful algal blooms (HABs) in such systems is increasing. The larger number of HABs identified is attributed to factors as eutrophication, climate change or more intense scientific monitoring (Carmichael, 2001). In freshwaters, cyanobacteria are responsible for almost all of the documented HAB events (Hudnell et al., 2008) and therefore are considered important factors when evaluating water quality in many countries (Chorus, 2005).

Cyanobacteria are very widely distributed around the Globe and are a typical constituent of phytoplankton in most freshwater ecosystems (Whitton and Potts, 2000). The explanation of factors leading to cyanobacterial dominance and massive development has been subject of a great number of studies. Importance of nutrient enrichment and unbalanced N:P ratios has been often suggested as a key factor, but unambiguous conclusions have not been reached, especially when none of these two nutrients are limiting (Oliver and Ganf, 2000). Strong stratification of the affected waterbodies may be of importance, as in non-turbulent systems floatability may offer great advantages to the cyanobacterial community (Oliver, 1994). Still, none of these factors can explain cyanobacterial dominance on their own, but a cobination of these and other factors may trigger cyanobacterial growth.

Beside the ecological impact of cyanobacterial dominance, their capacity to produce a great diversity of toxic compounds is of great interest. Most cyanobacterial toxins described so far are alkaloids or peptides (Falconer, 2005). Considering the second group, microcystins (MC) and nodularins are both cyclic peptides that cause severe damage on hepatic and gastric systems (Dawson, 1998). These cyclic peptides are possibly the best known cyanobacterial toxins and are responsible for most of the cyanobacterial poisonings described (Carmichael, 2001). Carcinogenic activity of MC had been suggested from early on (Falconer et al., 1989), and lately data from China and Serbia have both shown statistical correlation between appearance of hepatic cancer and consumption of MC affected water (Yu, 1989, 1995; Svircev et al., 2009). Considering the toxic alkaloids, cylindrospermopsin (CYN) is a toxin acting on a variety of targets, as it appears to enter cells readily (Chong, 2002; Falconer and Humpage, 2006). Irreversible inhibition of protein synthetase has been attributed to CYN (Froscio et al.,

2003), as well as genotoxic and carcinogenic activity (Humpage et al., 2000; Falconer and Humpage, 2001). The group of neurotoxic alkaloids includes anatoxins and saxitoxins. The anatoxins comprise two chemically very different compounds: anatoxin-a (ANTX-A), an acetylcholine analogue and anatoxin-a(s), an organophosphate. Anatoxin-a is a nicotinic agonist acting as a neuromuscular blocking agent, leading to muscle over-stimulation resulting in fatigue and paralysis (Carmichael et al., 1979; Dow and Swoboda, 2000) Anatoxin-a(s) acts in a biological different way, as it inhibits acetyl-cholinesterase, with equally lethal effects (Mahmood and Carmichael, 1986). Finally, the saxitoxins, which are better known from marine dinoflagellates (Steidinger, 1993), are able to inhibit transmission of nervous impulses by blocking sodium channels, such inhibition leading to death by respiratory arrest (Caterall, 1980)

Toxic cyanobacterial blooms producing one or more of these toxins have been described all over the world (Carmichael, 2008), although the distribution of the diverse types of cyanobacterial toxins were not homogeneous. Microcystins are the dominating cyanotoxins in freshwaters in most regions (Falconer, 2005), and a usual phenomenon in many countries. In Europe, diverse studies from Germany (Fastner et al., 1999), Denmark (Henriksen and Moestrup, 1997), France (Vezie et al., 1997) or Portugal (Vasconcelos, 1994) revealed that more than 60% of the analysed samples hosted MC. But MC are not an European phenomenon, massive presence has also been described for example in Asia (South Korea: Park et al., 1998) or America (United States: Graham et al., 2004). This MC-dominance is possibly not taking place in many regions of Australia, where CYN has been considered the major cyanotoxin (Burch and Humpage, 2005) since first being found responsible for the Palm Island Mistery Disease (Bourke et al., 1983).

Data on the occurrence of ANTX-A are generally scarce. High incidence has been observed in countries as Germany (Bumke-Vogt et al., 1999), Ireland (James et al., 1997) or South Korea (Park et al., 1998), while the studies driven in Finland, Spain or Italy (Sivonen and Jones, 1999; Carrasco et al., 2006; Messineo et al., 2009) showed very low incidence of this toxin.

3.1.3. Materials and Methods

Samples used in this study are of different origin, resulting from both the collaboration of diverse institutions and samplings carried out by ourselves. Special attention has been paid in order to guarantee that all samples were collected, processed and analysed following the exactly same procedures.

Samplings were performed from the year 2003 to 2009, and samples were either collected offshore or at the shore, avoiding possible scums, if not stated otherwise. Samples were obtained from seven different Spanish catchment areas. From the year 2006 on, fluorometric determination of algal groups was performed on a Moldaenke bbe Algae Analyser on fresh samples. Additionally, samples were GF/F filtered. Filters and, in some cases, filtrates were then stored frozen (-20 ºC) for toxin quantification.

Microcystins and anatoxin-a were extracted from filters by sonication into 90% and 100% methanol respectively. Cylindrospermopsin was extracted in acidified saline solution (0.9% NaCl, 5% formic acid) by ultrasonication (Branson 450 Sonifier). Extracellular MC and CYN were previously concentrated by solid phase extraction. For MC, C18 cartridges (Varian Mega Bond Elut C18, 1g) were used, aqueous methanol (90%) being used as eluent. Cylindrospermopsin was concentrated on graphitized carbon cartridges (Varian Bond Elut Carbon, 500 mg) as described in Wörmer et al. (2009). Before analysis, samples were dried down under vacuum and resuspended in 70% methanol (MC), 100% methanol (ANTX-A) or Milli-Q water (CYN). Quantification of the toxin was carried out by HPLC-PDA or LC-MS/MS. Concerning HPLC-PDA, the system consisted of a Waters Separations Module 2695, equipped with a Waters 996 PDA. For MC determination, chromatographic conditions as described by Lawton et al. (1994) were employed, using a Purospher STAR RP-18 endcapped 5µm 4.6 mm x 250 mm column. Chromatograms were monitored at 238 nm and toxin concentration determined by comparison to the injected standards for MC-LR, -RR and -YR. Anatoxin-a was detected by HPLC-PDA as described by Edwards et al. (1992) on a Luna (2) 3µm 4.6 x 100 mm column. Cylindrospermopsin determination was achieved on a Waters Spherisorb ODS2 5µm 4,6 mm x 250 mm column according to the protocol described by Törökné et al. (2004), chromatograms being monitored at 258 nm. When needed, HPLC-PDA analysis of MC and CYN were confirmed by LC-MS/MS. Also, samples from the summer 2009 were analysed by LC-MS/MS. In this case, the system

consisted of a Varian 500-MS IT Mass Spectrometer supported by two Varian 212 LC chromatography pumps and a 410 autosampler. Chromatographic separation was achieved with a Pursuit 3 C18 2 x 150mm column, mobile phase was Milli-Q water (A) and methanol (B). For microcystins, 0.2% formic acid and 5mM ammonium formate were added. Gradient (%A/%B) applied for MC was 60/40 to 0/100 in 18 minutes and for CYN100/0 to 50/50 in 10 minutes. MS/MS conditions are detailed in table 3.1.1

	Nebulizer gas pression (psi)	Drying gas pressure (psi)	Drying gas temperature (ºC)	Precursor Ion	Capilary Voltage (V)	Escitation Amplitude (V)
MC-RR	50	30	300	519.7	60	0.9
MC-YR	50	30	300	1045.3	185	1.8
MC-LR	50	30	300	995.4	140	1.5
CYN	65	35	300	416	110	1.05

Table 3.1.1 MS/MS conditions used for detection of three MC variants and CYN

Concerning the different natural samples obtained, intensive sampling was performed in Santillana reservoir (40º 42' 28''N 03º 49' 02''W), Valmayor reservoir (40º 31' 39''N04º 03´ 19''W), Trasona reservoir (43º 32' 33''N 05º 52' 35''W), Cogotas reservoir (40º 43' 15''N 04º 41' 42''W) and the pond in Parque de Juan Carlos I (40º 27' 57''N 3º 36' 09''W). In these cases, sampling frequency varied between one and two weeks. Except for the temporal evolution of cyanobacterial presence in these systems, monthly averaged values have been used.

Additionally, 74 sampling points at 58 waterbodies in seven watersheds were sampled on a monthly basis or once along the summer. The geographical location of these watersheds is shown in figure 3.1.1, Cantábrico, Miño-Sil and Galicia-Costa watersheds are summed up in the Norte basin.

Figure 3.1.1. Catchment area distribution in Spain (Ministerio de Medio Ambiente, Rural y Marino, Spain)

3.1.4. Results

Considering overall presence of cyanobacteria in the studied waterbodies, expressed as chl *a* concentration attributed to cyanobacteria by flurometry, a wide range of values may be observed (fig. 3.1.2). In more detail, 35% of the samples showed very limited presence of cyanobacteria (< 1 μg l^{-1}). A similar amount of samples showed chl *a* concentrations of cyanobacteria ranging between 1 and 10 μg l^{-1}. Finally, high (10 to 50 μg l^{-1}) and very high (>50 μg l^{-1}) presence was detected in 18 and 10% of the samples respectively. To further study these data, observations were grouped in corresponding watershed. A first impression shows that the Guadiana, Tajo and Norte watersheds seem to be hosting largest cyanobacterial presence.

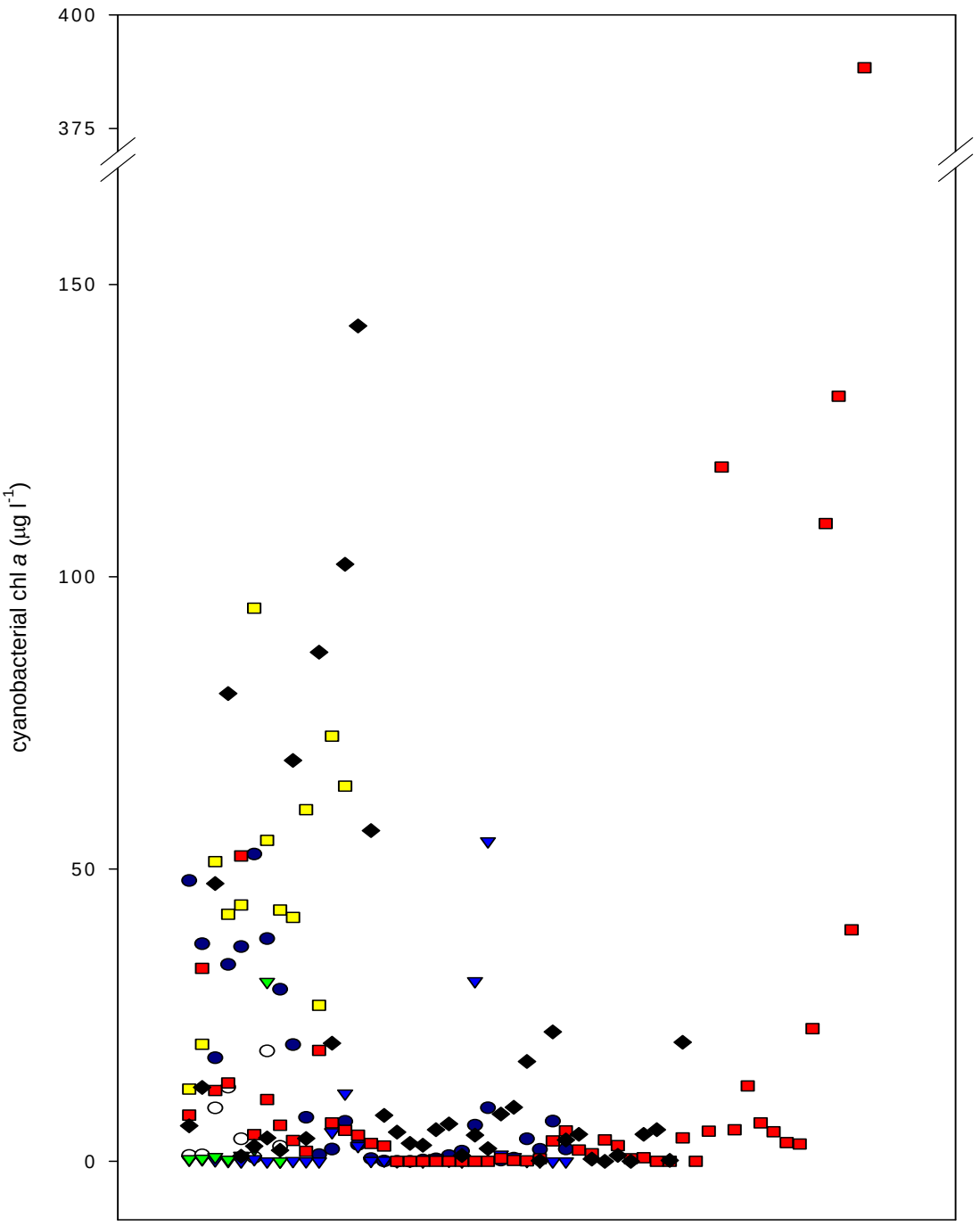
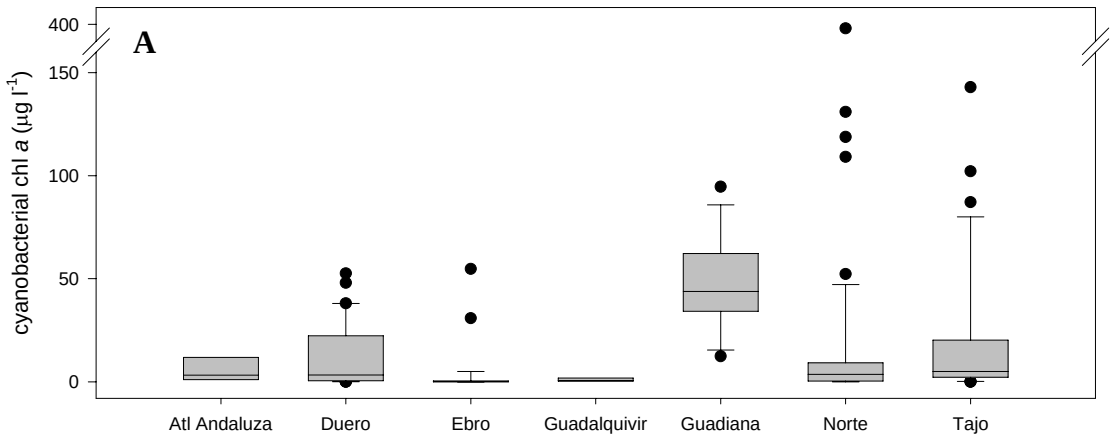


Figure 3.1.2. Cyanobacterial chlorophyll a concentration in Spanish freshwaters. Data are grouped in seven drainage basins: Atlántica Andaluza (white circle), Duero (blue circle), Ebro (blue triangle), Guadalquivir (green triangle), Guadiana (yellow square), Norte (red square) and Tajo (black diamond)

In order to allow a more correct analysis of these data, box plots including median and 10^{th}, 25^{th}, 75^{th} and 90^{th} percentiles were performed for each watershed considering chl a concentration of cyanobacteria (fig. 3.1.3 A) and ratio of cyanobacterial chl *a* vs. total chl *a* (fig. 3.1.3 B). The first plot confirms the extraordinary high presence of cyanobacteria in the waterbodies studied at the Guadiana watershed, median value being 43.86 μg l^{-1}. In the other basins, median values are much lower, remaining below 5 μg l^{-1}. Both Duero and Tajo watersheds are characterised by a great distance between median and 75^{th} and 90^{th} percentile, thus suggesting the existence of a large number of waterbodies affected by high presence of cyanobacteria. In the cases of Ebro, and especially, Norte watersheds the existence of some outliers, which suggest massive presence of cyanobacteria under very specific circumstances, have to be considered. The second plot (fig. 3.1.3 B) shows that the relative importance of cyanobacteria is high in almost all watersheds. In five of the cases studied, median importance of cyanobacteria is above 34%. In Guadalquivir watershed, cyanobacteria account for a median value of 25%, while in Ebro watershed their median presence is almost neglectable (5%), except, as said before, for some very specific episodes. Further, considering the dispersion of data, it is interesting to observe that some watersheds show very wide box plots, as is the case of Atl. Andaluza, Duero, Norte or Tajo watersheds, importance of cyanobacteria being quite variable. Other watersheds show much narrower distributions, the most interesting is possibly Guadiana.



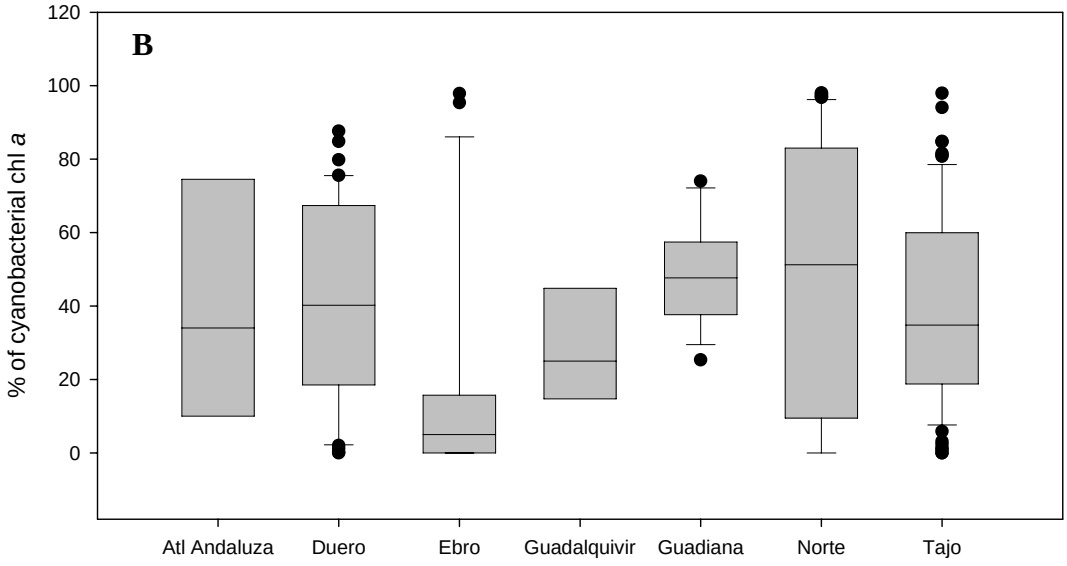


Figure 3.1.3. Cyanobacterial chl a (A) and percentage of cyanobacterial on total chlorophyll a (B) in seven Spanish watersheds. Box plots represent median, 10th, 25th, 75th and 90th percentiles and outliers.

Temporal evolution of cyanobacterial presence along the period June-October was studied for these watersheds. Therefore, data from waterbodies which fulfilled the following conditions were used: a) availability of at least three datapoints from three different months in one year b) cyanobacterial chl *a* concentration of at least 0.5 μg l^{-1} at some time. Figure 3.1.4 shows the obtained graphs for all watersheds, Atlántica Andaluza had to be excluded from this representation, as no complete datasets were available. The samplings performed at Santillana and Cogotas reservoir have been represented separately in order to allow a better graphical representation of the data from Tajo and Duero watersheds respectively. Generally speaking, period of maximum cyanobacterial development seems to be located between August and October. In the northern regions, as Norte, Ebro or Duero watersheds, period of maximum occurrence seem to be later (September-October) than in the more southern regions (Guadalquivir, Guadiana, Tajo), in which largest cyanobacterial communities seem to be observed in late August.

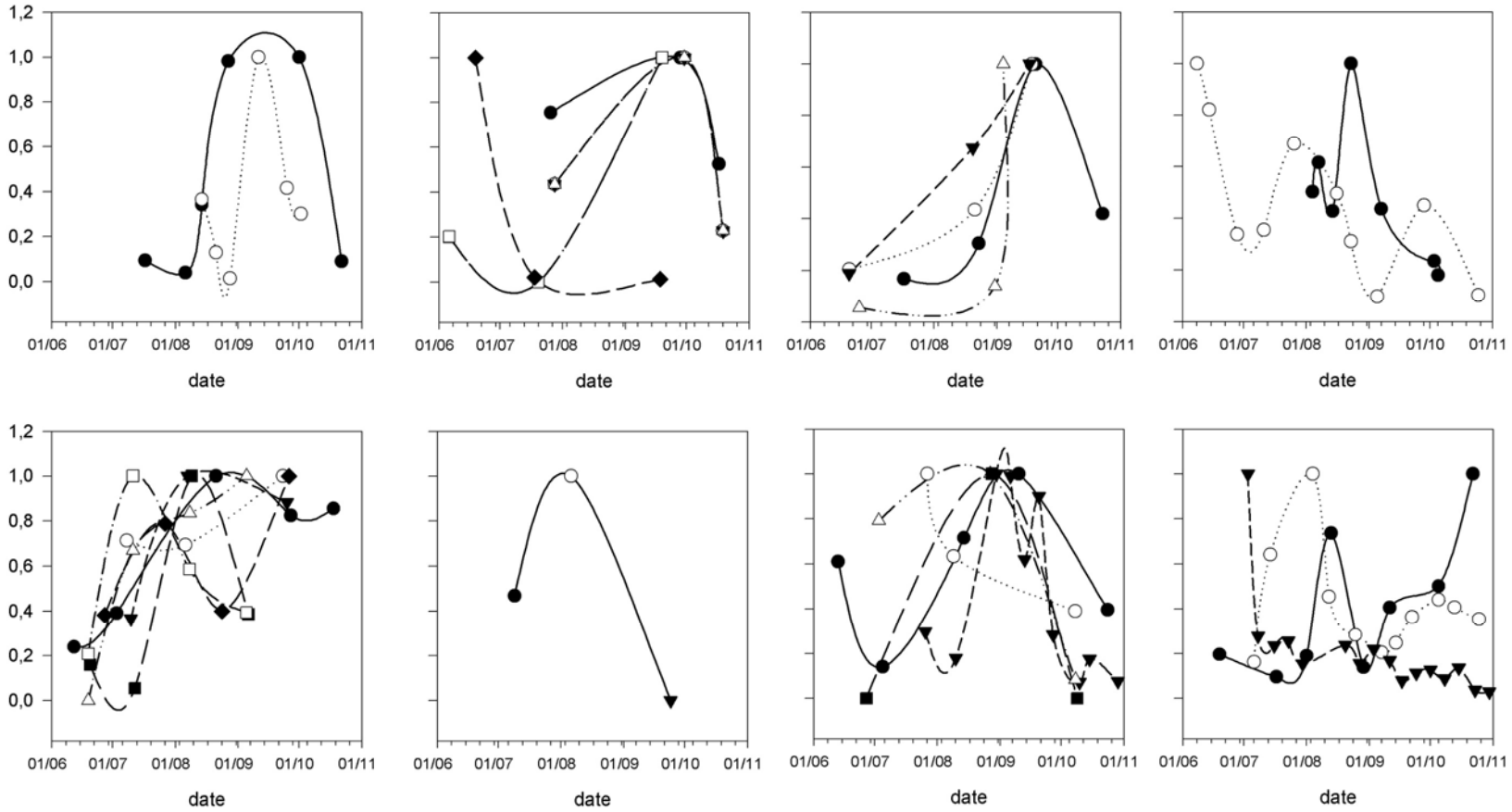


Figure 3.1.4. Temporal evolution of cyanobacterial chlorophyll a in the studied basins. First row, from left to right: Norte basin, Ebro basin, Duero basin without Cogotas reservoir, Cogotas reservoir. Second row: Guadiana basin, Guadalquivir basin, Tajo basin without Santillana reservoir, Santillana reservoir. Represented data have been previously normalized by dividing the values of each data set with the maximum value of the data set

Concerning cyanobacterial toxins, MC, CYN and ANTX-A were analysed. Table 3.1.2 shows the number of sampling sites and waterbodies in which these toxins were analysed, as well as the positive results observed in each case. Microcystins were detected in 50% of the studied waterbodies, while only 10.5% and 5% of these waterbodies were respectively affected by CYN and ANTX-A.

	Sampling sites		**Waterbodies**	
	Tested	Positive	Tested	Positive
Microcystin	53	25	34	17
Cylindrospermopsin	51	4	38	4
Anatoxin	74	3	60	3

Table 3.1.2: Cyanotoxin occurrence in Spanish freswaters

In the case of CYN, as identification by HPLC-PDA is not always unmistakable, presence was confirmed by LC-MS/MS. For example figure 3.1.5 shows the MS/MS spectrum for dissolved CYN recovered by SPE from Encinarejo reservoir, which contains the chracteristic product ions for CYN.

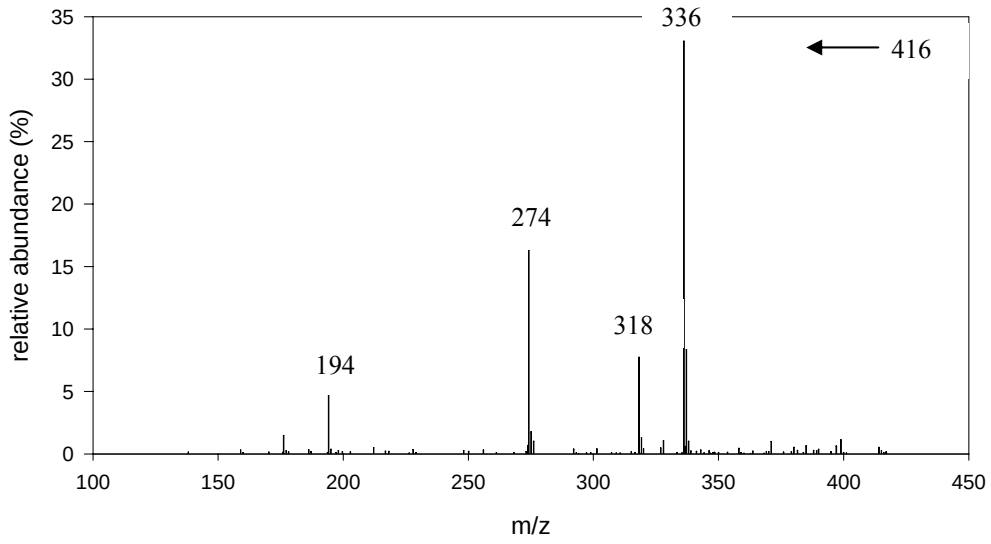


Figure 3.1.5: MS/MS product ion spectra of the [M+H$^+$] ion of CYN from Encinarejo reservoir (Jaén, Spain)

Figure 3.1.6 shows maximum toxin concentration values observed in each of the affected waterbodies. Anatoxin-a concentration was extremely low in the positive samples, being below our quantification limit in two cases and showing a maximum concentration of 0.31 μg l^{-1} in the third reservoir. The case of CYN, although also only a few positive samples are available, is clearly different. In this case, sestonic concentration of up to 16.1 μg l^{-1} are observed, and in all positively tested waterbodies, maximum sestonic CYN concentration is above 2 μg l^{-1}. Finally, maximum MC concentrations are quite variable in the different waterbodies. Highest MC concentration in the studied samples was 277 μg l^{-1}. In scum samples, up to 2.7 mg l^{-1} were detected. Still, most of the positively tested reservoirs show very low MC concentration, sestonic concentration remaining below 1 μg l^{-1} in 53% of the cases.

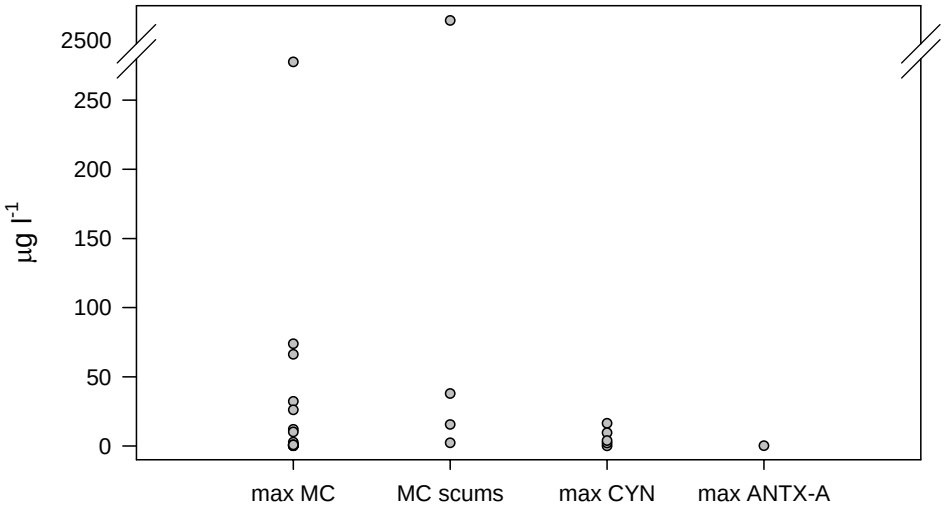


Figure 3.1.6. Maximum cyanotoxin concentration in waterbodies positively tested for these toxins

Concerning toxin vs. cyanobacterial chl *a* ratios (figure 3.1.7), in the case of MC, most values are found in the range of 0.1 to 1 μg MC / μg chl *a*. Nevertheless, ratios as high as 4.8 are also observed. In the case of CYN, relative toxin production is lower. Maximum CYN/chl a ratio found in our studies is 0.34. In both cases, dispersion of data pairs is quite wide, not following any concrete pattern.

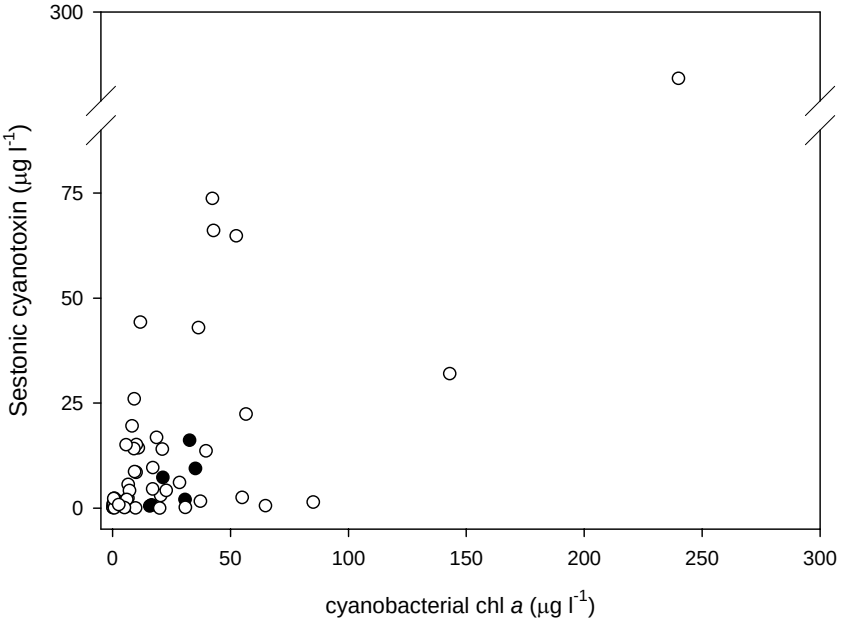


Figure 3.1.7. Data pairs representing cyanobacterial chl a and sestonic cyanotoxin content: microcystins (white) and cylindrospermopsin (black)

Until here, only sestonic, intracellular toxins have been considered, but dissolved, extracellular toxins should also be taken into account when evaluating cyanobacterial toxin occurrence. Figure 3.1.8 represents data pairs of sestonic and dissolved MC and CYN. Very clear differences are easily established, MC tending towards the intracellular fraction and CYN being strongly represented in both fractions. Highest extracellular MC concentration detected was 0.77 μg l^{-1}, which was 1.2% of concurrent sestonic MC concentration, while highest extracellular CYN concentration was 7.83 μg l^{-1}, a value three times larger than the concurrent sestonic concentration.

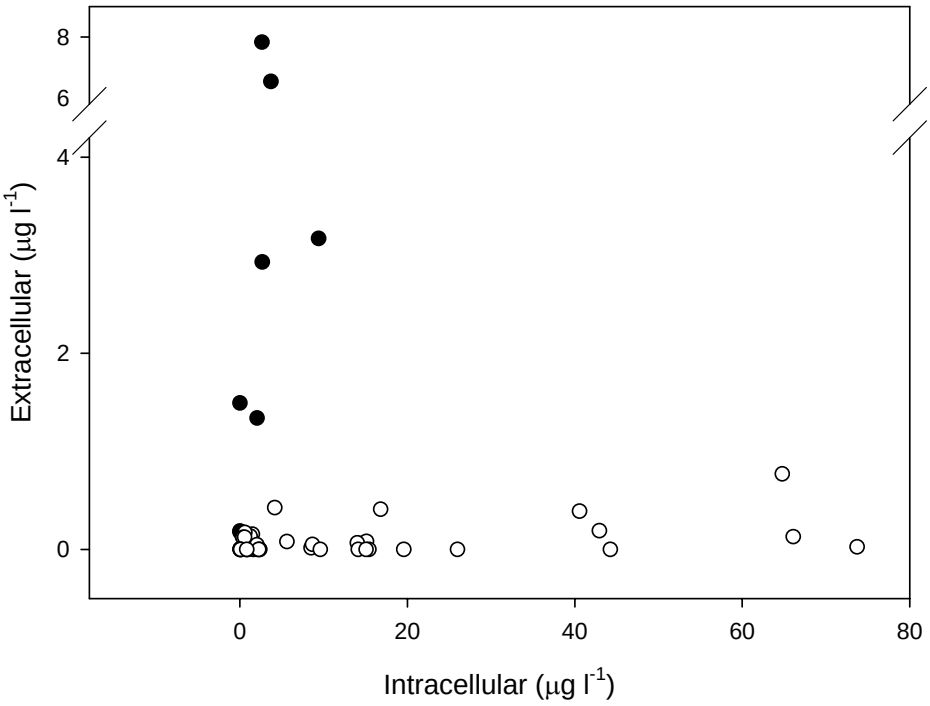


Figure 3.1.8. Data pairs representing intra- and extracellular microcystin (white) and cylindrospermopsin (black)

3.1.5. Discussion

Potentially toxic cyanobacteria have been confirmed as a menace for Mediterranean reservoirs in the last decades (Vasconcelos, 1994; Vezie et al., 1997; Cook et al., 2004, Messineo, 2009). Also in Spain, local or regional studies have demonstrated that both cyanobacteria and cyanotoxins may be considered a matter of concern (Quesada et al., 2004; De Hoyos et al., 2004; Aboal and Puig, 2005; Carrasco et al., 2006). In this study, we tried to approach a more global understanding of the problem in Spanish freshwaters, taking into account cyanobacteria and the three most common cyanotoxins: microcystins, cylindrospermopsins and anatoxin-a. Further, regional and temporal distributions were considered. It has to be noted that the obtained results may show some kind of bias towards increased cyanobacterial presence, as some of the samplings were specifically performed in systems where a potential risk due to cyanobacterial blooms was expected. To minimize the impact of these data, monthly averaged values have been used and occurrence has been related to studied reservoirs, and not to analysed samples.

Spanish watersheds are usually hosting cyanobacteria during summer and autumn. This is consequent, for example, with the observations of Zohary et al. (2004) in Lake Kinneret (Israel). Even though heavy blooms are already detected in June, for example in Cogotas or Santillana reservoir, major cyanobacterial growth is generally observed from August to October. Our data further allow differentiating time-periods of maximum cyanobacterial development in different watersheds. Generally speaking, it seems as if cyanobacteria grow later in the northern catchment areas (Norte, Ebro, Duero) than in the southern (Guadalquivir, Guadiana), the very large Tajo catchment area showing an intermediate behaviour. This phenomenon may be related to higher temperatures allowing an earlier and more solid stratification of Southern reservoirs, as such stratification results beneficial for cyanobacteria and their capacity to regulate floatability. In waterbodies with frequent heavy cyanobacterial blooms, as Cogotas or Santillana reservoir, seasonality of cyanobacterial presence is harder to establish. In these cases, conditions are possibly very benefitial for cyanobacteria during the whole summer and autumn, thus allowing massive growth at any timepoint and, in some cases, more than one bloom episode. The correct understanding of most probable bloom-periods may be of interest when designing watershed management policies.

Photo 3.1.1: Images of cyanobacterial blooms in (from left to right): Cueva Foradada, Arcos and Oliana reservoir and of a watersampler with sample from Cogotas reservoir

Besides the temporal distribution of cyanobacteria, the regional characterization of seven watersheds was considered interesting. We observed that cyanobacteria were very important in almost all of them, usually comprising about one third of the phytoplanktonic community, with the only exception of the Ebro watershed. In this case, even though concrete episodes of massive cyanobacterial blooms were detected, median cyanobacterial / total chl *a* ratio was only 5%. The waterbodies sampled in the Guadiana watershed are clearly the most strongly affected by cyanobacteria, not only median chl *a* concentration is above 40 μg l^{-1} and median cyanobacterial / total chl a ratio above 47%, but also intrabasin variability is lower than in any other watershed. In watersheds like Atl. Andaluza, Duero, Norte or Tajo, variability is very large, thus suggesting the coexistence of cyanobacteria-dominated systems and systems in which cyanobacteria are almost absent.

The cyanobacterial communities described before are responsible for the production of MC, CYN and ANTX-A. Microcystins are clearly the most abundant cyanotoxins in Spanish freshwaters, having been detected in 50% of the sampled reservoirs. This MC-dominance has been described previously for reservoirs in Central Spain (Carrasco et al., 2006) and waterbodies worldwide (Sivonen and Jones, 1999). Another interesting aspect is that most of the MC-positive waterbodies show only low toxin concentration. Indeed, more than half of the reservoirs showed maximum MC concentrations below the WHO recommended limit of 1 μg l^{-1}. These data are mainly explained by the large number of reservoirs in which cyanobacteria are present only in small concentration, as toxicity of the biomass, expressed as MC/chl *a*, is quite high in most cases, in fact 82% of the samples show a ratio above 0.1.

Both CYN and ANTX-A were also detected in Spanish waterbodies during the survey performed. In the case of ANTX-A only three waterbodies were tested positively, and in only one of them the toxin concentration was above our quantification limit. This very low incidence of ANTX-A in Spanish waters had been described previously by Carrasco et al. (2006) and our data only confirm this tendency. Also, recent data from Italy (Messineo et al., 2009) seem to confirm that ANTX-A is unusual in the Mediterranean area. Nevertheless, anatoxin-a has been reported as a usual toxin in Germany (Bumke-Vogt et al., 1999) or South Korea (Park et al., 1998) and concrete

ANTX-A containing episodes have been described for example in Poland (Pawlik-Skowronska et al., 2004) or Ireland (James et al. 1997).

Cylindrospermopsin was also detected in only a few occasions, concretely in 10.5% of the tested waterbodies. In this case though, concentration was much higher, maximum concentration in all of the CYN containing waterbodies being higher than 2 $\mu g\ l^{-1}$. Cylindrospermopsin was typically known from tropical or subtropical regions (e.g.: Banker et al., 1997; Chapman and Schelske, 1997, Shaw et al., 1999), and considered absent from European waters. In this last decade, though, CYN detection is experiencing a rapid increase and today it has been described for the first time in Germany (Fastner et al., 2003), Italy (Manti et al., 2005), Spain (Quesada et al., 2006), Finland (Spoof et al., 2006), Poland (Kokocinski et al., 2009) Czech Republic (Bláhová et al., 2009) or France (Brient et al., 2009) . Possibly, the limited number of positively tested waterbodies and the high CYN concentration in all of them might indicate that the analytical tools for most samples, HPLC-PDA, could not be able to detect low environmental CYN concentration. Possibly, the use of more sensitive analytical techniques, as LC-MS/MS, will allow detection of CYN in a larger number of samples, as is the case in the studies performed by Fastner et al. (2006).

One peculiarity of CYN is that large proportions of the toxin can be present extracellularly, both in laboratory cultures (Saker and Griffiths, 2000, Preussel et al., 2007) and natural samples (Wörmer et al., 2009 and references within). Our data clearly confirm this tendency and also show the great differences between CYN and MC regarding the importance of extracellular toxin concentration, an aspect that should be considered seriously when developing management policies.

In this study, we have been able to describe the extended occurrence of both cyanobacteria and cyanobacterial toxins in Spanish freshwaters, and especially in regions as the Guadiana, Duero or Tajo basins. August, September and October have been identified as the months most suitable for cyanobacterial maxima, major growth taking place earlier in the southern basins. Finally, microcystins are confirmed as a great matter of concern, as 50% of the studied waterbodies were affected by this toxin. But also cylindrospermopsin has to be considered seriously, especially due to the very high concentrations found in the dissolved fraction.

3.1.6. Acknowledgement

This work was made possible by the FPU fellowships (Ministerio de Educación, Spain) awarded to L. Wörmer and S. Cirés. We are very grateful to Ministerio de Medio Ambiente, Rural y Marino (Spain), CEDEX (Ministerio de Fomento, Spain), Confederación Hidrográfica del Norte (Spain), Confederación Hidrográfica del Ebro (Spain) and Canal de Isabel II (Spain) for funding and providing samples.

4. Advances in solid phase extraction of the cyanobacterial toxin cylindrospermopsin

Cyanobacterial toxins may occurr in a wide range of concentrations. Even low doses may pose a risk to human health and ecosystem quality, may serve as an indicator of the risk of future toxic blooms or may help us understand their fate in the environment. Therefore, especially when available detection methods do not offer enough sensibility, previous concentration steps may be necessary.

In the case of of intracellular toxin, large amounts may be obtained by processes as filtration or centrifugation. In the case of extracellular toxin, dissolved in the aqueous phase, such accumulation of large amounts is not as easily achieved. Solid phase extraction has proven useful for a variety of compounds, and here we tried to optimize a method for the recovery of cylindrospermopsin, a toxin which may be expected in relatively high concentration in the extracellular phase.

We considered it mandatory to obtain a method that would allow optimal recoveries both from environmental samples and experimental enclosures in which concentration was very low and from culture medium, in which very high amounts of CYN could be expected.

4.1. Advances in solid phase extraction of the cyanobacterial toxin cylindrospermopsin

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Abstract

Unlike other cyanotoxins, cylindrospermopsin (CYN) is often found in high concentrations in the extracellular fraction, possibly due to a higher release from the producing cells and a limited degradation once liberated. This fact demands effective recovery methods from environmental samples to guarantee a proper quantification. A reliable and simple solid phase extraction (SPE) method was obtained by the sole use of graphitized carbon cartridges and by paying special attention to both the selection of the most suitable solvent and the need of sample preparation prior to SPE. An acidified combination of dichloromethane and methanol not only showed best recoveries, but also allowed drastic reduction to the elution volumes needed with complete recovery being achieved in only 8 mL. Acidification of the sample and addition of sodium chloride are suggested as valuable improvements to the SPE method and turn out to be essential for correct and robust recovery of CYN in environmental samples of great diversity in terms of pH, DOC, and conductivity. Excellent behavior of the proposed method over a wide range of CYN loadings, applied sample volumes, and DOC loadings was also confirmed.

Introduction

Cylindrospermopsin (CYN) is a cyanobacterial alkaloid consisting of a tricyclic guanidine moiety combined with a hydroxymethyluracil initially described as a potent hepatotoxin (Ohtani et al. 1992). Toxic effects have been observed on diverse organisms. For example, and as a result of the studies carried out by Kinnear et al. (2007), potential impacts on amphibian populations and, thus, far-reaching ecological impacts have been predicted. Also bioaccumulation has been observed, for example, in the freshwater mussel *Anodonta cygnea* (Saker et al. 2004). In vertebrates, the main target of CYN seems to be the liver. Still, other organs such as the thymus, kidney, adrenal glands, lungs, intestinal tract, and heart may also be affected. Besides other episodes, CYN has been implicated as cause of hepatoenteritis on Palm Island, Australia, affecting 148 people (Bourke and Hawes 1983; Bourke et al. 1986). Further, assays have shown CYN-induced genotoxicity (Humpage et al. 2000, 2005; Shen et al. 2002) and evidence for carcinogenity (Falconer and Humpage 2001).

Several cyanobacterial species are able to produce cylindrospermopsin. Among these species, the most widely distributed is *Cylindrospermopsis raciborskii*, which is now found in many countries (Briand et al. 2004). Other species producing this cyanotoxin are *Umezakia natans* (Harada et al. 1994), *Anabaena bergii* (Schembri et al. 2001), *Raphidiopsis curvata* (Li et al. 2001), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Aphanizomenon flos-aquae* (Preussel et al. 2006), *Anabaena lapponica* (Spoof et al. 2006), or *Lyngbya wollei* (Seifert et al. 2007).

Even though the mechanisms of CYN release from the producing cells are still not completely understood, recently Mihali et al. (2008) suggested CyrK to be a transporter for CYN. In the field, the observation of high extracellular concentrations has been distinctive for this toxin. In this way, Rücker et al. (2007) observed that in 31% of samples collected from twenty-one lakes, dissolved CYN was more than 80% of total CYN. Also in laboratory cultures, a similar behavior has been confirmed by Norris et al. (2001) for a *Cylindrospermopsis raciborskii* culture or Shaw et al. (1999) in the case of *Aphanizomenon ovalisporum*.

The common presence of CYN in the dissolved fraction demands effective methods of extraction and concentration, both for the purpose of recovery of toxin from spent medium as well as for the correct quantification of toxin in field samples.

Norris et al. (2001) performed a complete study with a range of SPE cartridges and observed best results when using

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4.1.1. Abstract

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4.1.2. Introduction

Cylindrospermopsin (CYN) is a cyanobacterial alkaloid consisting of a tricyclic guanidine moiety combined with a hydroxymethyluracil initially described as a potent hepatotoxin (Ohtani et al., 1992). Toxic effects have been observed on diverse organisms. For example, and as a result of the studies carried out by Kinnear et al. (2007), potential impacts on amphibian populations and thus far-reaching ecological impacts have been predicted. Also bioaccumulation has been observed, for example in the freshwater mussel *Anodonta cygnea* (Saker et al., 2004). In vertebrates, the main target of CYN seems to be the liver; but other organs such as the thymus, kidney, adrenal glands, lungs, intestinal tract and heart may also be affected. Besides other episodes, CYN has been implicated as cause of hepatoenteritis on Palm Island, Australia, affecting 148 people (Bourke and Hawes, 1983; Bourke et al., 1986). Further, assays have shown CYN-induced genotoxicity (Humpage et al., 2000, 2005; Shen et al., 2002) and evidence for carcinogenity (Falconer and Humpage, 2001).

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Even though the mechanisms of CYN release from the producing cells are still not completely understood, recently Mihali et al. (2008) suggested CyrK to be a transporter for CYN. In the field, the observation of high extracellular concentrations has been distinctive for this toxin. In this way, Rücker et al. (2007) observed that in 31% of the samples collected from twenty-one lakes dissolved CYN was more than 80% of total CYN. Also in laboratory cultures, a similar behaviour has been confirmed by Norris et al. (2001) for a *Cylindrospermopsis raciborskii* culture or Shaw et al. (1999) in the case of *Aphanizomenon ovalisporum*.

The common presence of CYN in the dissolved fraction demands effective methods of extraction and concentration, both for the purpose of recovery of toxin from spent medium as especially for the correct quantification of toxin in field samples.

Norris et al. (2001) performed a complete study with a range of SPE cartridges and observed best results when using graphitized carbon. Metcalf et al. (2002) combined graphitized carbon with C18 and obtained good recoveries when working with high concentrations of CYN. In the present study, we focused on further optimization of a graphitized carbon based SPE method, paying special attention to both solvent selection and sample preparation, aspects which so far had been avoided. Our goal was to obtain a reliable method which not only enables bulk recovery of CYN from spent medium but which would allow optimal concentration of even small amounts of CYN from environmental samples.

4.1.3. Materials and Procedures

Aphanizomenon ovalisporum UAM 290 was grown and harvested when achieving the stationary phase for the obtention of CYN. Cells were separated from the spent media by centrifugation. Both supernatant and pellet were further used as CYN source in the different experiments. Cell bound CYN in the pellet was extracted with 0.9% saline solution containing 5% formic acid by pulse-pestle ultrasonication on a Branson 450 Sonifier. Ultrasonication was applied in 70% duty cycles during two minutes at maximum energy recommended for micro-tips. CYN in the supernatant was GF/C filtered and stored (-20 ºC) for further use.

The different recovery experiments were all performed in triplicate and graphitized carbon cartridges (Bond Elut Carbon 500 mg, Varian Inc.) were used. These cartridges were primed with the selected solvent and washed with distilled water. Afterwards the sample was applied at a flow rate of 5 ml min^{-1} and a further washing step with distilled water and the final elution with the selected solvent were performed. A volume of 10 ml was chosen for each of the priming, washing and elution steps described above, sample volume was 100 ml unless specified otherwise. Finally, the eluent was vacuum-dried and prepared for HPLC analysis.

Quantification of CYN was performed on a Waters Alliance 2695 HPLC system with a 996 PDA detector equipped with a Waters Spherisorb 5µm ODS2 column according to the protocol described by Törökné et al. (2004). The presence of CYN was verified by its UV spectrum and its retention time and quantified with the help of a five-point calibration curve.

All statistics were performed with the XLSTAT software (Addinsoft SARL).

4.1.3.1 Development of the SPE method: selection of solvents and sample preparation

Norris et al. (2001) suggested methanol as the solvent selected for elution of CYN and emphasized the importance of acidification of the solvent. In the present study we also tested the suitability of acetone and combinations of dichloromethane:methanol (4:1, 1:1, 1:4) as solvents. In all cases these solvents were acidified with 5% formic acid (v/v). For the selection of the solvent, cartridges were prepared as described above and extracted CYN in saline solution containing 5% formic acid was applied to the cartridges. The eluent was collected in 2 ml fractions and analysed. Once an appropriate solvent had been selected, importance of sample preparation was studied. Instead of using CYN extracted from the cell bound fraction into acidified saline solution, the toxin was obtained from the dissolved fraction naturally present in the spent medium.

In order to test the possible benefits of acidification, the pH of this dissolved fraction was adjusted respectively to values of 2, 4, 7 and 10, using formic acid and NaOH (0.1 N). Also, the importance of the ionic strength in the sample was tested. Used concentrations were 0, 0.1, 0.2, 0.4 and 0.9% sodium chloride (w/v).

4.1.3.2. Suitability of the SPE method for CYN in environmental samples and culture medium

To test the suitability of the sample preparation and extraction method proposed, samples with increasing presence of CYN (0.1 to 30 µg) were applied to the cartridges. The extent of the recovery for the diverse quantities added was used to estimate the linearity of the response. Further, the importance of sample volume and DOC concentration was also studied. In order to test the suitability of the method for increasing sample volumes, 1 µg CYN was added to 10, 100, 500 and 1000 ml distilled water and applied to the cartridges. In the case of the studies concerning sensitivity to

DOC, CYN had to be recovered from 100 ml distilled water to which humic acid (Acros Organics) had been added to final concentrations of 0, 100, 250, 500 and 1000 mg l^{-1} DOC.

Afterwards, the fact of working with natural samples of great diversity was considered. GF/C filtered water from five waterbodies was spiked with CYN. The waterbodies selected for this study were four reservoirs: Arcos (Cadiz, Spain) Cogotas (Avila, Spain), Salas (Orense, Spain) and Vega de Jabalón (Ciudad Real, Spain) and the pond in Parque Juan Carlos I (Madrid, Spain). Subsurface water was used in all cases except Cogotas reservoir, for which samples from 18m depth were used. In both Arcos reservoir and the pond in Parque de Juan Carlos I (JC I) CYN has been detected recently (Quesada et al., 2006; Wörmer et al., 2008). Prior to spiking, dissolved organic carbon (DOC), pH and conductivity were determined in the samples, DOC being quantified on a Shimadzu TOC 5000. Before applying the spiked water (CYN = 0.89 μg) to the activated cartridges, half the samples were acidified with formic acid 1% (v/v), and sodium chloride was added (0.1% w/v), while the other half were applied without further preparation. A combination of dichloromethane:methanol (1:4) with the addition of 5% formic acid was selected as solvent. In parallel, these environmental samples were also analysed without external addition of CYN.

Finally, the capacity of the proposed method to guarantee good recovery of CYN in a wide range of concentrations was tested. Therefore, a declining culture of *Aphanizomenon ovalisporum* UAM 290 was sonicated by pulse-pestle ultrasonication on a Branson 450 Sonifier in the spent medium and centrifuged (3000 g, 15 min). The supernatant was GF/F filtered and applied to the cartridges as described above. 1784 μg were applied in a sample volume of 200 ml, effluents containing non retained CYN were collected at 10 ml intervals. Afterwards, CYN retained in the cartridge was recovered according to the proposed method.

4.1.4. Assessment

4.1.4.1. Development of the SPE method: selection of solvents and sample preparation

Percentual recoveries achieved by the use of different solvents are shown in fig. 4.1.1. The use of acetone showed the worst recovery values of all solvents tested, in 20

ml only 43% of the initially added CYN was eluted. The use of methanol clearly improved the recoveries observed, achieving a value of 88%. Finally, the addition of dichloromethane to the methanol further improved the obtained results. Not only higher recoveries were achieved (98%), but also the needed elution volume was drastically reduced.

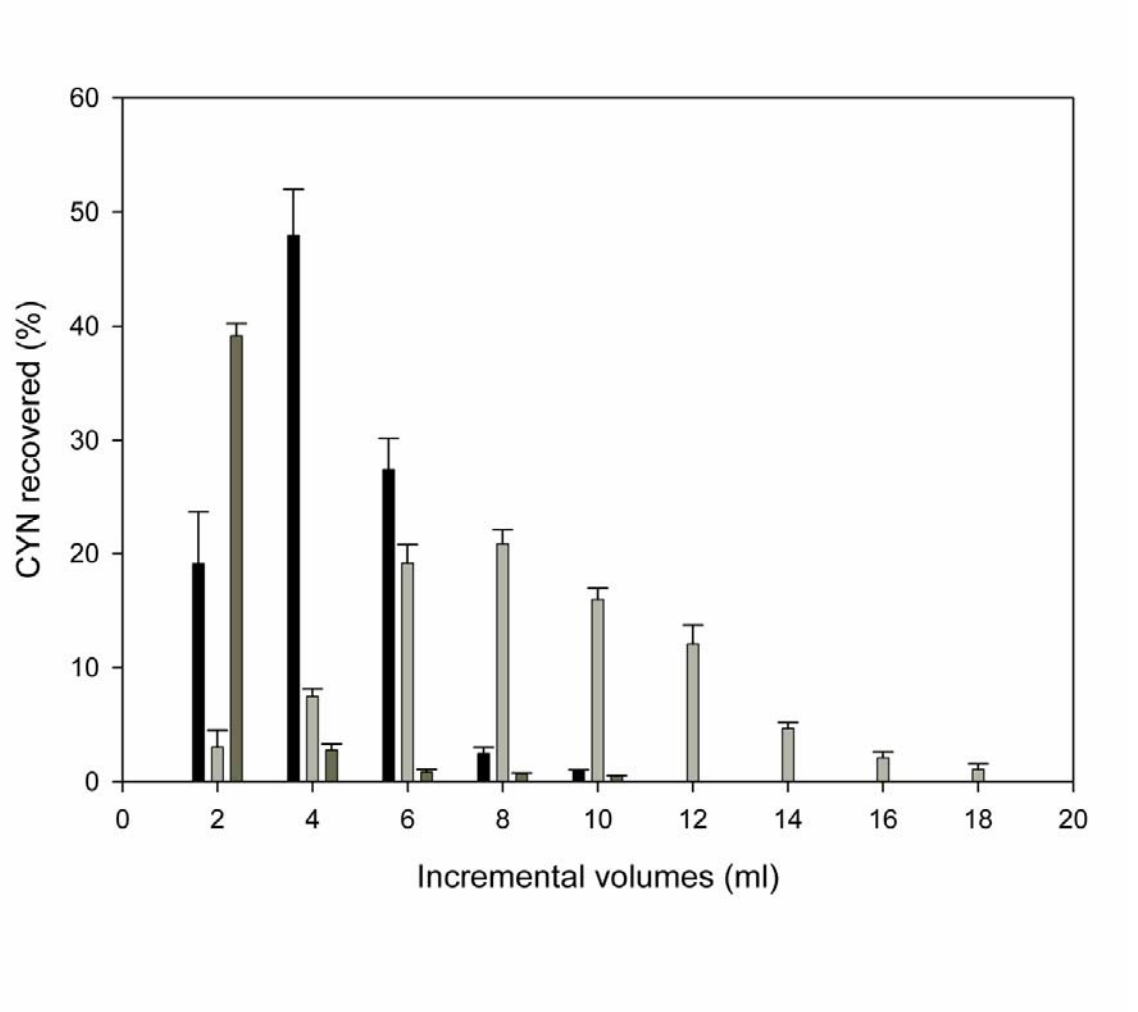

Figure 4.1.1: Elution profile of CYN applied to graphitized carbon cartridges. Different solvents were tested: dichloromethane:methanol (4:1), black bar; methanol, light grey bar; acetone, dark grey bar. All solvents were acidified (5% formic acid). Mean and standard deviations are represented (n=3)

Once the benefits of adding dichloromethane to methanol were established, different combinations of both solvents were tested, always in presence of 5% formic acid. The three combinations tested (dichloromethane:methanol, 4:1, 1:1 and 1:4) showed similar recoveries and elution profiles. Beside this, an improvement in the chromatographical resolution of the peaks was observed as the percentage of dichloromethane decreased (data not shown).

The importance of acidifying the sample to be passed through the cartridge is shown in table 4.1.1. Recoveries are not significantly different (t-Student, $\alpha = 0{,}05$), although slight improvements may be observed at lower pH, up to 96.4% of the initial CYN added was retrieved. Also, it should be noted that an increased tailing of the chromatographical peak was observed when the samples were not acidified (fig. 4.1.2), in some cases the importance of this tailing was as high as 15% of total peak area (fig. 4.1.2 B).

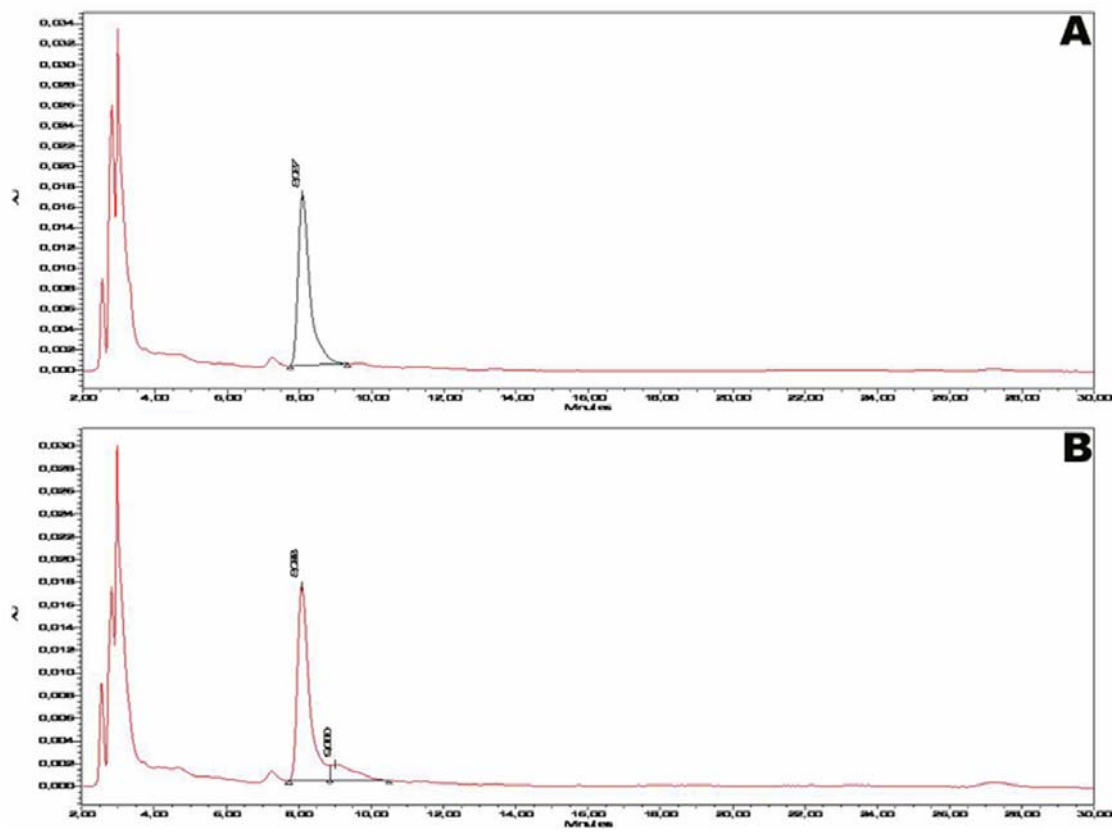

Figure 4.1.2: HPLC chromatograms of CYN recovered by SPE. Extracellular CYN (2.07 µg) in spent medium was used and pH was adjusted to values of 2 (fig. A) and 7 (fig. B) before applying to the cartridge.

Also, the importance of concentration of sodium chloride was evaluated (table 4.1.1). Although only small differences in the recovery values were observed, the presence of sodium chloride seemed to be related with more robust measurements as expressed by decreasing standard deviations, the differences observed being statistically significant (t-Student, $P = 0.002$). For concentrations above 0.1% neither additional effects nor further improvement could be described.

pH	NaCl concentration (% w/v)	CYN recovery (%)
10	0	94.6 ± 1.87
7	0	94.6 ± 2.81
4	0	96.4 ± 1.66
	0	96.1 ± 2.23
	0.1	96.2 ± 0.68
2	0.2	94.8 ± 0.94
	0.4	95.8 ± 0.75
	0.9	96.3 ± 0.74

Table 4.1.1: Effect of sample preparation by modification of pH and/or addition of sodium chloride on recovery of CYN from graphitized carbon cartridges (n=3), mean and standard deviation are shown. pH values of 4 and 2 are achieved by the addition of 0.01% and 1% formic acid (v/v) respectively.

4.1.4.2. Suitability of the SPE method for CYN in environmental samples and culture medium

Excellent linearity between CYN applied and recovered was achieved for quantities between 0.1 and 30 μg CYN (fig. 4.1.3). Also, recovery of 1 μg CYN from samples with volumes increasing from 10 to 1000 ml was very stable, and remained above 97% for all volumes tested (data not shown). Concerning the importance of DOC (fig. 4.1.4), loadings as high as 25 mg DOC did not affect the recovery of CYN by our method. Loadings of 50 mg seem to have slight negative impact, as recovery drops to 85.3%. Extremely high DOC loadings (100 mg) result in a more evident negative impact, as recovery was only 73.9%.

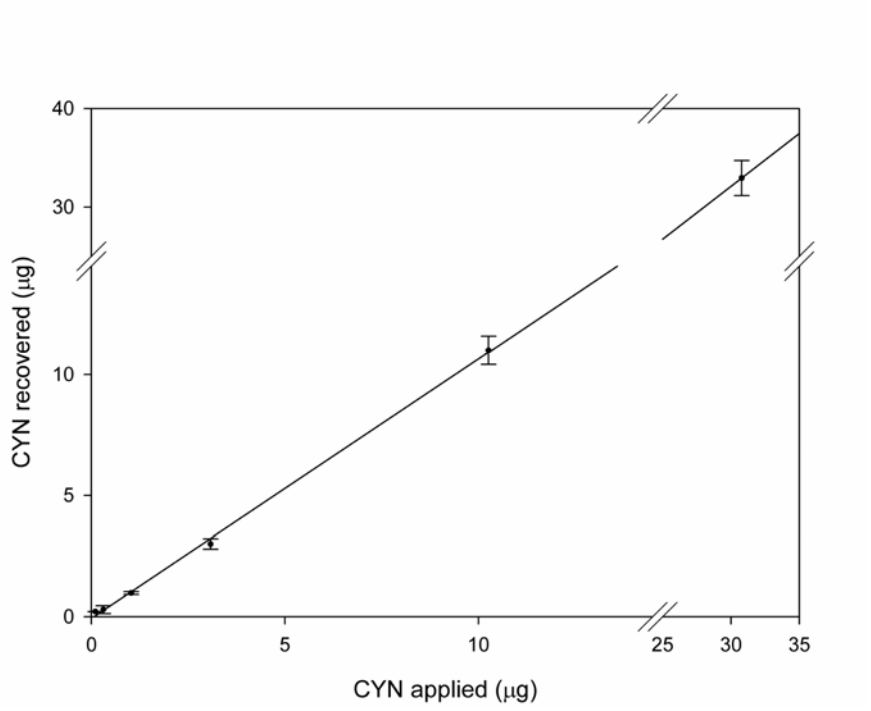

Figure 4.1.3: Recovery of increasing quantities of CYN applied to SPE cartridges. Mean and standard deviations are represented (n=3). Linear regression (R^2 = 0.999) is described by the equation y = 1,0691*x

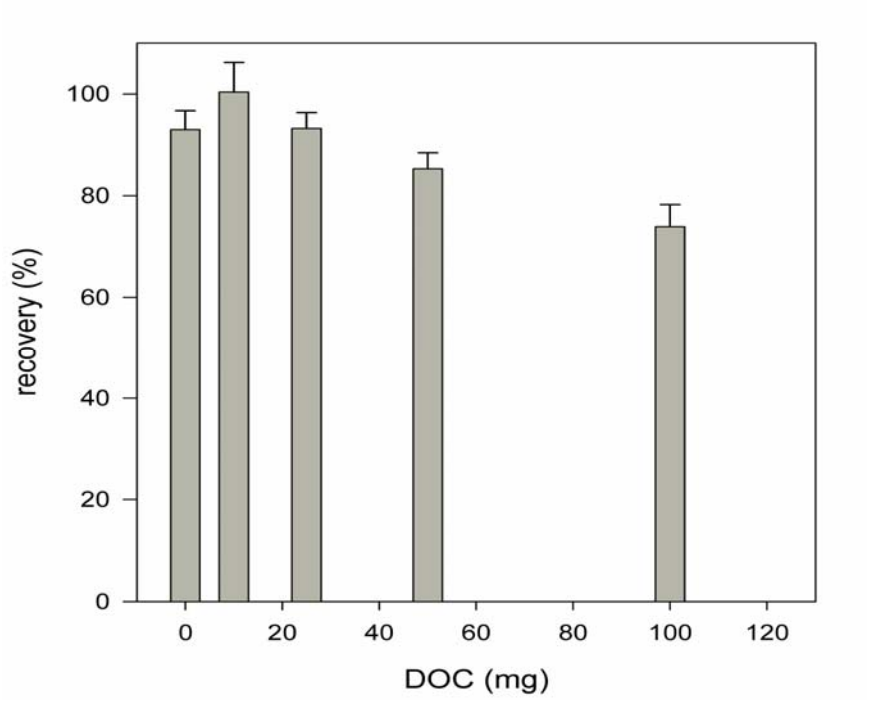

Figure 4.1.4: Recovery of CYN applied to SPE cartridges in samples with increasing presence of DOC. Mean and standard deviations are represented (n=3).

Before spiking the natural waters with CYN, the environmental samples selected were characterised (table 4.1.2). Salas reservoir shows lower conductivity and slightly acid waters while the other waterbodies are characterised by higher conductivity and pH. In the case of Cogotas reservoir the sample shows a higher pH, but conductivity remains low. DOC concentrations were quite high and ranged from 12.8 mg l^{-1} in Cogotas reservoir to up to 34.5 mg l^{-1} in Salas reservoir.

Concerning the recovery of CYN in these spiked samples (table 4.1.2), on the one hand, results were always above 90% when the samples were acidified and sodium chloride was added. Best recoveries were achieved for samples from Parque Juan Carlos I (JC I) and Cogotas reservoir, while in Arcos reservoir the worst recoveries (91.8%) were obtained. Naturally occurring CYN was only detected in the pond in Parque Juan Carlos I, concentration after SPE was determined to be 8.41 μg l^{-1}. The recovery values for this waterbody when working with spiked samples were obtained by subtracting the naturally occurring amount of CYN in the 100 ml sample from the total amount recovered.

On the other hand, when the sample was applied to the cartridges untreated, recovery values clearly decreased (table 4.1.2). In Arcos reservoir for example, recovery dropped to 80.2%, which is 11.6% less than with sample preparation. Only in Salas reservoir, where the natural pH is lowest (pH=6.04), no differences in recovery were observed (t-Student, P = 0,813). Differences in the recovery values were statistically significant for all samples (t-Student, α = 0,05) except for the above mentioned Salas reservoir and for Vega de Jabalón reservoir (t-Student, P = 0,094)

	Date of sampling	Depth of sampling	Conductivity (µS $cm^{-1}s^{-1}$)	pH	DOC (mg l^{-1})	CYN recovery (1% formic acid, 0.1% NaCl added)	CYN recovery (untreated sample)
Cogotas	28/06/2007	18 m	170	8.37	12.8 ± 0.05	0.95 ± 0.05 (106,6 %)	0.83 ± 0.04 (93.5%)
Salas	18/02/2007	subsurface	65	6.04	34.5 ± 0.08	0.87 ± 0.09 (97.9%)	0.89 ± 0.02 (99.8%)
Arcos	26/09/2007	subsurface	1320	7.94	24.6 ± 0.6	0.82 ± 0.06 (91.8%)	0.71 ± 0.03 (80.2%)
JC I	05/09/2005	subsurface	608	9.12	30.9 ± 0.7	0.87 ± 0.05* (98.3%)	0.77 ± 0.04* (86.7%)
Vega de Jabalón	03/07/2007	subsurface	1394	7.89	18.6 ± 0.3	0.86 ± 0.06 (97.4%)	0.78 ± 0.03 (87.8%)
JC I unspiked	05/09/2005	subsurface	608	9.12	30.9 ± 0.7	8.41 ± 0.34 **	7.60 ± 0.27 **

Table 4.1.2: Characterisation of the water samples used to test the suitability of the method proposed and recovery of CYN (n=3). Recovery is expressed as recovered µg CYN and as percentage of initial CYN spiked (0.89 µg) (*) Recovery obtained by subtracting the naturally occurring CYN amount from total recovery (**) CYN concentration in µg l^{1} is shown

When establishing the natural concentration of dissolved CYN without sample preparation, the toxin could also only be detected in the pond in Parque de JC I, and also here important differences were observed between the two methods studied. Without sample preparation, a concentration of 7.6 μg l^{-1} was calculated, which is 10% less than what was obtained when the sample was previously treated.

Finally, the capacity of our method to extract high amounts of CYN from culture medium was tested (table 4.1.3). For loadings as high as 1517 μg, no CYN could be detected in the effluent. Further loading of the cartridges results in non retained toxin being observed in the last three effluent fractions. Still, the sum of CYN in these fractions accounts for only 1.00 μg (0.056 % of overall CYN loaded). Afterwards, CYN retained by the cartridge was eluted following the proposed method, 1515 μg CYN were regained in only 10 ml of solvent.

Fraction	CYN fed (μg)	CYN in effluent (μg)	Ratio effluent:feed (%)
1 - 17	1517	n.d.	n.d.
18	1606	0.120	0.13
19	1695	0.313	0.35
20	1784	0.568	0.64

Table 4.1.3. Retention capacity of the SPE method for CYN, effluent is collected in 20 fractions and analysed. Effluent:feed ratio is calculated considering CYN fed and CYN in effluent in each fraction (n.d.: not detected).

4.1.5. Discussion

Field studies carried out all over the world in different kind of waterbodies have shown that very high proportion of total CYN can be found in the dissolved fraction (table 4.1.4). This accumulation in the extracellular fraction has not been completely explained yet, but could be related to an increased CYN release from the cells and a

long persistence of the toxin in the dissolved state due to limited degradation (Chiswell et al., 1999; Smith et al., 2008; Wörmer et al., 2008).

This high presence in the dissolved state demands efficient and robust methods for quantification. While quantification methods are well developed (Eaglesham et al., 1999; Törökné et al., 2004), concentration and extraction of dissolved CYN from natural samples has not been widely developed or published. Norris et al. (2001) completed a first approach towards an effective SPE method by selecting graphitized carbon as the most suitable compound when compared to a wide range of the most usual SPE sorbents. Also, in their work, the need for acidification of the solvents was established. Finally, when applying culture medium used to grow *Cylindrospermopsis raciborskii* to graphitized carbon cartridges with bed weights of 300 mg, up to 861 µg CYN was retained, thus showing the great capacity of these cartridges for CYN. In the present study, we assumed these results but studied if an improvement of recoveries and in the elution profile could be obtained by the use of other solvents and conditions. While acetone was clearly not useful, the addition of dichloromethane allows to maximize recoveries and to reduce elution volumes.

At this stage, attention should be paid to the differences in the elution volume for methanol obtained in our work when compared to those from Norris et al. (2001) and Metcalf et al. (2002), where total recovery was obtained in about 8ml, half the volume observed in the present study. An explanation to this different behaviour may be found in the bed weight of the cartridges. We used cartridges of 500 mg graphitized carbon, while Norris et al (2001) used 300 mg, in Metcalf et al. (2002) no indication about the quantity used could be found. According to diverse manufacturers, the 500 mg cartridges will allow loadings of over 1 g of analyte and are recommended for large sample volumes (100 ml – 1 l). Low concentration of the toxin in field samples usually demands higher volumes to be passed through the SPE cartridges. Thus, in our opinion, the larger cartridges are more suitable for natural samples, and should be considered as standard method for routine analysis. Therefore we consider the modification of solvents proposed here essential to allow correct concentration and quantification of the toxin in environmental samples.

Sampling site	Number of samples	Dissolved CYN (μg l^{-1}) [% of total CYN]	Particulate CYN (μg l^{-1})	Source
Hervey Bay water storage facility, 1997 - 98 (Australia)	8	7 – 63 [19 – 95%]	0.7 – 29	Chiswell et al., 1999
Palm Lakes, 1997 (Australia)	2	22 – 120 [85-100%]	0 – 4	Shaw et al. 1999
Ocean Park, pond 1, 1997 (Australia)	2	14 – 16 [87,5 – 100%]	0 – 2	Shaw et al. 1999
Ocean Park, pond 2, 1997 (Australia)	1	4 [100%]	0	Shaw et al. 1999
Aquaculture pond, 1997 (Australia)	1	39 [6,6%]	550	Saker and Eaglesham, 1999
NE German lowlands, 2005 (Germany)	115 (21 lakes)	0 – 11.8	0 – 0.5	Rücker et al., 2007
Parque de Juan Carlos I, 2005 (Spain)	3	2.93 – 7.83 [52 – 75%]	2.63 – 3.7	Wörmer et al., 2008
Arcos reservoir, 2004 - 05 (Spain)	4	0.18 – 3.17 [25 – 63%]	0.54 – 9.4	Quesada et al. (2006) and unpublished data

Table 4.1.4. Data on the occurrence of particulate and dissolved cylindrospermopsin

Also, Norris et al. (2001) and the later work from Metcalf et al. (2002) did not explore the possible need of some previous sample preparation. But in this work, we have been able to demonstrate the improvement achieved by acidification and addition of sodium chloride, when working with natural samples of diverse characteristics. Even though acidification and addition of sodium chloride did not result in great improvements when applied to CYN present in spent medium – possibly due to peculiar characteristics of growth media concerning for example high concentration of salts – they were essential for accurate recoveries in spiked environmental samples and in the unspiked sample from the pond in Parque de Juan Carlos I. When sample preparation was avoided, up to 11.6% less CYN was recovered from spiked samples, and even more important, without sample preparation, CYN concentration in an environmental sample was estimated to be 10% lower than if sample was treated. These losses were apparently related to samples that showed pH values above 7, as shown in the fact that only in the case of Salas reservoir (pH = 6.04), recoveries were similar. As the presence of phytoplankton in water, and thus the consumption of CO_2 via photosynthesis, will increase pH values, toxic blooms can be expected to occur mainly in waters with high pH. It should be noted that – at least in Spain – CYN has been observed only in basic waterbodies, namely Arcos reservoir and the pond in Parque de Juan Carlos I (Quesada et al., 2006; Wörmer et al., 2008). Also the appearance of CYN producing organisms in Shaw et al. (1999) or Seifert et al. (2007) are related to sites presenting pH values above 7. Sample preparation thus seems to be most needed in the kind of waterbodies that mainly have been described to house CYN producing organisms.

Also, it should be noted that the high concentrations of DOC present in the spiked environmental samples did not affect recovery of CYN, the proposed SPE method remained reliable at DOC values of up to 34.5 mg l^{-1}. This is consequent with our experimental results (fig. 4.1.4) that show that loadings of up to 25 mg DOC do not affect the SPE of CYN and that loadings of even 50 mg DOC have only very small negative effect on recovery of CYN. This is important as graphitized carbon shows extraordinary adsorption of organics to its surface and consequently problems with the extraction of compounds from samples with high DOC could be expected. These saturation effects have not been found in our experiments, possibly due to the larger bed weight in the cartridges, which guarantees an increased adsorption surface.

Another aspect that should be pointed out is that the proposed method shows very good linearity when comparing applied to recovered CYN for quantities ranging from 0.1 to 30 µg. This indicates that on the one hand small amounts of toxin can be correctly concentrated and recovered and on the other hand risk of breakthrough of the cartridges should be very low for environmental samples. Small deviations when applying 0.1 µg CYN can be explained due to chromatographic limitations. As recovered samples are concentrated in 1 ml saline solution, but only 100 µl are injected, quantification is being made at the limit of our system (10 ng CYN injected to the chromatography column). The sample volume applied to the cartridges has shown to have no impact at all on the recovery of CYN, therefore, when working with HPLC systems similar to those used in the present study, we suggest sample volumes to be at least 500 ml. This allows correct detection of CYN concentrations close to the provisional standard for drinking waters of 1 µg l^{-1} and in samples with DOC concentrations ranging as high as 50 or even 100 mg l^{-1}.

Finally, the excellent capacity of the proposed method to recover high amounts of toxin is remarkable. In our study (table 4.1.3), 1784 µg CYN were loaded, and the sum of non retained CYN in effluents accounts for only 0.056% of this amount. In their experiments, Norris et al. (2001) were able to retain up to 861 µg CYN on SPE, calculated as the difference in feed and effluent, in our case retention increased to more than two fold that amount. It has to be noted that in the case of Norris et al. (2001) such recoveries are achieved with high breakthrough already occurring. In our case, breakthrough is only starting, concentration in the final effluent fraction is only 0.64% of feed concentration. Besides studying the retention of CYN, we were also interested in the ability to efficiently regain this high amount of toxin. In only 10 ml, up to 1515 µg were recovered (84.9% of CYN in feed). The use of cartridges with larger bed weights, together with the selection of the most appropriate solvents seem to be responsible for this high retention capacity and massive recovery .Our data thus clearly show that very substantial amounts of CYN can be easily extracted from culture medium and regained in small volumes. It should be considered that all results are obtained by the sole use of graphitized carbon cartridges. Avoiding the use of an additional C 18 cartridge, as proposed by Metcalf et al. (2002), is a further advantage in terms of optimal time and resource management.

Therefore, for analysis of dissolved CYN in environmental samples and recovery from growth medium, we suggest a SPE method consisting of a sample preparation with 1% formic acid and 0.1% sodium chloride and the use of a combination of dichloromethane:methanol (1:4) acidified with 5% formic acid as solvent for the graphitized carbon cartridges used.

4.1.6. Comments and Recommendations

Solid phase extraction by the sole use of graphitized carbon cartridges is confirmed as a suitable method for the concentration of CYN from culture medium or from very diverse environmental samples. The use of a solution of methanol:dichloromethane as eluent is strongly recommended, as it allows the use of increased bed weights while guaranteeing to achieve optimum recoveries in small elution volumes. Eluents used so far, namely methanol, result in an excessive broadening of the elution profile, thus complicating total recovery.

Previous sample preparation, namely acidification and addition of sodium chloride, has been avoided so far, but turns out to be essential for correct and steady recovery of CYN from very diverse environmental samples, and thus for correct analysis of water quality. Excellent response over a wide range of CYN concentrations, sample volumes and DOC loadings further backs the method proposed.

Considering analysis of CYN in other environmental samples, and especially in drinking water, influence of treatment processes on the method should be studied separately.

4.1.7. Acknowledgements

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5. Degradation of the cyanobacterial toxins microcystin and cylindrospermopsin

Inside producing organisms, toxins are well protected from degradation. Once they are liberated to the waterbody though, different biotic and abiotic agents may be responsible for the degradation of these compounds and, in some cases, a loss in toxicity. If it takes place *in* situ, such degradation will be of great importance as it may, for example, help avoiding exposure to the toxin in recreational waters or in inadequately treated drinking water.

Two agents are expected to be mainly responsible for degradation of cyanobacterial toxins in the field: bio- and photodegradation. We studied the effect of both processess on microcystins and cylindrospermopsin. Special attention was paid to develop experimental setups mimicking natural conditions and to confirm the loss in toxicity attributed to degradation.

5.1. *In situ* microbial degradation of microcystins in two Spanish reservoirs.

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5.1.1. Abstract

Different variants of microcystins are shown to be efficiently degraded by bacterial populations from Valmayor and Santillana reservoirs (Madrid, Spain) during *in situ* microcosm experiments. Lag phases are observed and attributed to the availability of other organic compounds. Only as these are used up, microcystin consumption seems to be taking place. Further, attempts to isolate microcystin degrading bacteria were performed by serial enrichment steps in medium with microcystins as sole nitrogen, carbon and energy source. Finally, the bacterial consortia responsible for degradation of microcystins were tested for the presence of the *mlrA* gene. The gene could not be detected neither during the biodegradation experiments, nor in the subsequent enrichment steps or in isolated colonies. Our results therefore suggest the existence of modified versions of the *mlrA* gene or even the possibility of alternative gene clusters and enzymatic pathways involved in biodegradation of microcystins. Such findings might be of interest when genetic probes for rapid detection of MC-degrading bacteria in the environment are designed.

5.1.2. Introduction

Cyanobacterial massive occurrence has become usual in waterbodies throughout the world (Codd, 2000), this dominance being of concern because of the ability of some cyanobacteria to produce diverse toxins (Carmichael, 1992). The most common of these toxins, the microcystins (MC), act as potent hepatotoxins, inhibiting serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990). Tumour-promoting activity (Falconer, 1991), gastroenteritic and hepatic diseases and irritant reactions have been linked to the presence of microcystins (Dawson, 1998 and references within). Considering chronic exposure, a possible correlation between presence of toxic cyanobacteria in drinking water and an increased appearance of liver cancer has been reported in China (Yu, 1995). Ueno et al. (1996) confirmed this possible correlation to be occurring with low doses of microcystins, given that the highest concentration detected in drinking water was 0.460 μg l^{-1}. Recently, a connection between primary liver cancer and MC, as one risk factor for high incidence rate of this cancer, has been suggested in Serbia (Jukovic et al., 2008; Svircev et al., 2009).

Microcystins are accumulated by the producers as intracellular metabolites, liberated mainly by cell death and lysis. In laboratory experiments with *Planktothrix (Oscillatoria) agardhii,* Sivonen (1990) found that leakage of microcystins to the medium increased towards the end of the growth phase. Low extracellular MC concentration has also been described for *Anabaena* or *Microcystis* (Rapala et al., 1997; Wiedner et al., 2003). In environmental samples, concentration in the extracellular fraction generally remains one or more orders of magnitude below those of the sestonic fraction (e.g.: Poon et al., 2001; Oh et al., 2001). The large difference in concentrations between sestonic and soluble fractions can be attributed to dilution, adsorption to particles, bio- and photodegradation. In natural environments, biodegradation by autoctonous micro-organisms is suggested as the main pathway of decrease in microcystin concentration beside dilution (Sivonen and Jones, 1999). Therefore, *in situ* biodegradation processes will be extremely relevant for human health, as low MC doses can be critical, but also for the ecosystem. Hulot and Huisman (2004), for example, modelled the importance of degradation of toxins by heterotrophic bacteria on the outcome of competition between toxin-producing and toxin-sensitive phytoplankton species.

The experimental data concerning time needed for biological degradation of microcystins have been found to be somehow variable. Most authors observed that MC concentration decreased below the detection level in around a week (e.g.: Cousins et al., 1996; Christoffersen et al., 2002; Hyenstrand et al., 2003; Ishii et al., 2004), or slightly more (Takenaka and Watanabe, 1997; Holst et al., 2003). Kiviranta et al. (1991) found microcystin to be persistent over an experimental period of two months. It should be considered that the conditions under which these experiments were carried out, as for example initial microcystin concentration, use of crude or purified toxin or temporal and spatial origin of the inoculum vary strongly. The degradation curve for microcystins is generally described as exponential, although Jones and Orr (1994) observed a two-step degradation, starting from extraordinary high initial concentrations of dissolved microcystins (1300 $\mu g\ l^{-1}$). Moreover, previous exposure of the bacterial population to microcystins could enhance biodegradation (Rapala et al., 1994; Holst et al., 2003) in contrast to non exposed populations.

Even if biodegradation capacity seems to be naturally present in almost all systems affected by toxic cyanobacteria, actual degradation seems to be carried out only by a smaller subgroup of the bacterial population. Recently, more studies have become available concerning the identification of the organisms responsible for this biodegradation. Members of the genus *Sphingomonas* have most often been described as microcystin decomposing bacteria (Bourne et al., 1996; Saito et al., 2003; Ishii et al., 2004; Valeria et al., 2006). Also, Bourne et al. (2001) identified a gene cluster involved in biodegradation of MC-LR by *Sphingomonas*. Members of the genera *Pseudomonas* (Takenaka and Watanabe, 1997) and *Sphingopoyxis* (Ho et al., 2007a) have also been isolated and identified as MC-degrading bacteria.

Few studies have been published about the importance of other organic carbon sources on biodegradation of MC. Christoffersen et al. (2002) approached this aspect and described degradation of dissolved organic carbon (DOC) to be correlated with the degradation of microcystins, thus suggesting the coupling between these processes. Still, different results, as for example the existence of short lag phases, were observed when the MC source was a cyanobacterial lake community instead of laboratory cultures or purified MC.

Our aim was to simulate naturally occurring MC biodegradation by microcosm experiments under natural conditions and in presence of realistic amounts of MC in crude extracts from laboratory cultures and field samples. Further, attempts to isolate MC degrading bacteria were performed. Finally, we were interested in evaluating if the *mlrA* gene, described by Bourne et al. (2001) to be involved in the first step of MC degradation, could be detected in isolated colonies, and more importantly, in samples of bacterial assemblages in which biodegradation was actively taking place. A rapid detection of *mlrA* would allow an immediate and correct evaluation of the degradation potential of a given bacterial community, as suggested by Saito et al. (2003).

5.1.3. Materials and methods

Microcosm experiments were performed during October 2004 at Santillana reservoir and during October 2007 at Valmayor reservoir. Both waterbodies are located in Central Spain and have repeatedly been affected by toxic cyanobacterial blooms (Carrasco et al., 2006). In the summer before the deployment of the experimental setup, our data show that MC were present in both systems in sestonic concentrations as high as 73 and 26 μg l^{-1} respectively.

The experiments were performed in triplicate in low-density-polyethylene enclosures that served as microcosms. These were filled up with 15 l GF/F-filtered water from the reservoir. In Santillana reservoir, crude toxic extract was obtained from biomass collected from the reservoir in September, when the phytoplanktonic community was mainly composed by cyanobacteria, green algae and diatoms. The extract was obtained by serial extractions in methanol (90%) after sonication. The extract was then completely dried under vacuum, resuspended in distilled water, filter-sterilized (0.2 μm) and added to the enclosures to a MC concentration of 4.5 μg l^{-1} MC-LR. In Valmayor reservoir, the toxic extract was obtained after harvesting exponentially growing *Microcystis novacekii* UAM 247. In this case, extracts included MC-LR, -RR and -YR and samples were spiked to initial concentrations of 15.2 μg l^{-1}. Negative controls were obtained by autoclaving the GF/F filtered water (121 ºC, 20 min).

The enclosures were deployed into the reservoir at 4 m depth in order to minimize possible photodegradation, which according to our observations is restricted to surface layers of the water column. In the case of Santillana reservoir, incubation

lasted 12 days, samples for HPLC analysis were taken after 0, 4, 8 and 24 hours and then after 2, 4, 7 and 12 days; samples for bacterial counting after 1, 2, 4 and 12 days. For bacterial counting, the sample was diluted with sterilized distilled water (1:8) and filtered through a 0.22 μm Whatman Anodisc filter. These filters were stained with Yo-Pro 50 μM, following the procedure proposed by Xenopoulos and Bird (1997). Countings of at least 400 bacteria per sample were performed by epifluorescence microscopy. In the case of Valmayor reservoir, experimental period lasted 22 days, with sampling at days 2, 5, 8, 12, 15, 19 and 22. In this case, bacterial counting was performed by flow cytometry. Samples for flow cytometry were fixed with formaldehide to a final concentration of 2% (Lebaron et al., 1998) and stored in darkness at 4ºC. Staining with SYTO 9 obtained from Molecular Probes (LIVE/DEAD *Bac*Light Bacterial Viability Kit) was performed before analysis on a Beckman Coulter Cytomics FC 500 MPL cytometer. Calibration for quantification was performed by addition of beads in known concentration to the analysed samples. Excellent correspondence between values obtained by epifluorescence countings and by cytometry was confirmed.

Microcystin analyses from the microcosms experiment were performed by HPLC. As samples were initially spiked with low MC concentrations, previous concentration procedures were needed. Therefore solid phase extraction was employed, using a previously activated C18 cartridge (Varian Mega Bond Elut C18) and eluted from these cartridges with 90% aqueous methanol. This eluent was then concentrated by evaporation, resuspended and prepared for injection. Quantification of MC was achieved following the procedure proposed by Lawton et al. (1994). The HPLC-PDA System (Alliance, Waters) consisted of a Waters Separations Module 2695, equipped with a Waters 996 PDA. The chromatography column was a Purospher STAR RP-18 endcapped (5μm) 4.6 mm x 250 mm column. Chromatograms were monitored at 238 nm and toxin concentration determined by comparison to the injected standards for MC-LR, -RR and -YR.

In the experiments conducted at Valmayor reservoir, an attempt to isolate MC degrading bacteria was performed following the methods proposed by Valeria et al. (2006). After MC degradation was observed, samples from each enclosure were taken in triplicate at day 15 and diluted (1:5) in sterilized Mineral Salt Medium (MSM) to which

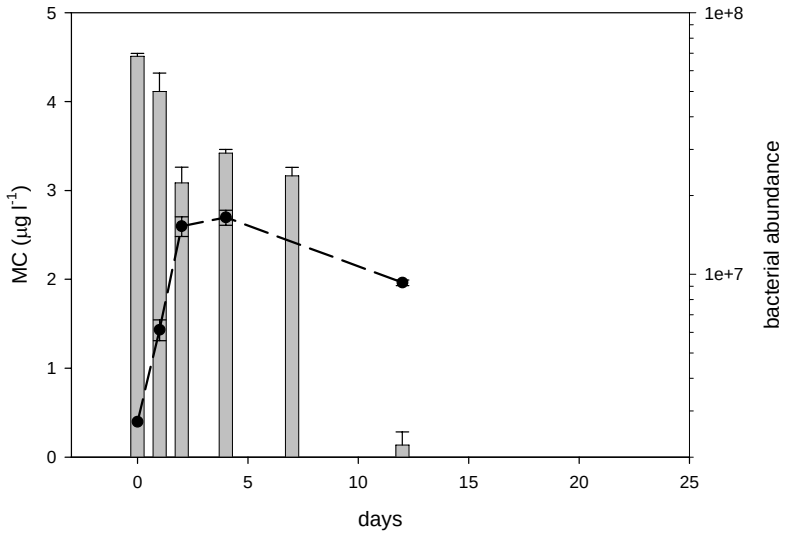
MC-LR had been added to a concentration of 250 μg l^{-1} as sole carbon and nitrogen source. Further enrichment steps were performed by 1:5 dilutions in new MC-spiked medium as soon as degradation was observed. The quantification of MC in the culture was performed by protein phosphatase inhibition assays (PPA) from ZEU Inmunotec (Zaragoza, Spain) in order to rapidly estimate the extent of degradation and to evaluate toxicity of possible degradation products. The accuracy of these measurements were afterwards confirmed by HPLC as described above. Finally, after the fifth enrichment step, an aliquot of the resulting subculture was transferred to one-tenth nutrient tryptone soy agar to which MC-LR had been added. In addition, another aliquot was transferred to Sakurai medium as proposed in Tsuji et al. (2006) and to PYE (0.5% beef extract, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% glucose, 1% agar). Single colonies were isolated from these plaques and inoculated into MSM medium supplemented with MC-LR (250 μg l^{-1}) in order to test their biodegradation capability. MC-LR concentration after 20 days of incubation was measured by HPLC methods as described before.

Finally, isolated colonies, as well as samples from the biodegradation experiment and the enrichment steps in MSM were tested for the presence of the *mlrA* gene, which Bourne et al. (2001) had found to be responsible for the first step in the degradation pathway they described in *Sphingomonas* sp.. Isolated colonies were directly picked up from the solid medium. Samples from the biodegradation experiment were obtained at days 8, 12 and 15 by filtration on Supor-200 membranes (Pall Corporation). Samples from each enrichment step were obtained prior to the next dilution by both filtration (Supor-200 membrane, Pall Corporation) and by centrifugation. PCR-testing was performed on a Bio-Rad MJ Mini Personal Thermal cycler following the methods proposed by Saito et al. (2003), primers as suggested in their work were obtained from Metabion International. *Sphingomonas* ACM-3962 was obtained from the Australian Collection of Microorganisms and served as positive control for the PCR method.

5.1.4. Results

Considering the results obtained in the microcosm experiments conducted at Santillana and Valmayor reservoir, MC biodegradation took place efficiently. Complete degradation was observed in about two weeks after initial lag phases that were slightly longer in the case of Valmayor reservoir (fig. 5.1.1). In the case of Santillana reservoir,

the first 48 hours were characterised by what seems a short lag phase and an initial drop in toxin content, from 4.51 μg l^{-1} to 3.08 μg l^{-1}, the disappearance rate being 0.696 (μg l^{-1}) d^{-1}. Afterwards degradation was arrested during a period of at least five days, during which MC concentration remained stable. Main degradation took place between days 7 and 12, final MC concentration being below 5% of the initial concentration, degradation rate during these days may be quantified at 0.606 (μg l^{-1}) d^{-1}, this means that around 19% of the present toxin is consumed each day. Concerning Valmayor reservoir, an initial 8-day lag phase was observed. Afterwards, degradation started and MC was reduced to 6.5% of initial concentration in only 7 days. During this period of main degradation, degradation rate for the sum of MC variants is 1.99 (μg l^{-1}) d^{-1}, in other words, about 13% of the toxin disappears each day.



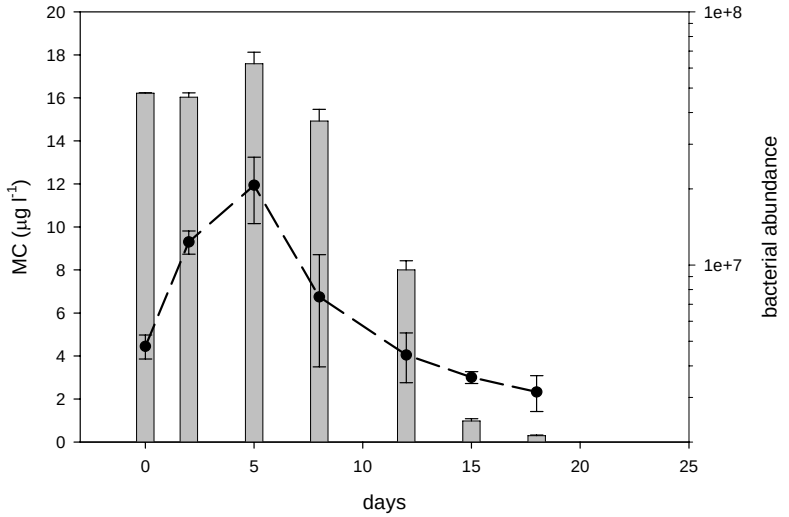


Figure 5.1.1. Biodegradation of microcystins (grey bars) and growth of the bacterial population (dashed line) during the Santillana 12 day-experimental period (A) and the Valmayor 22 day-experimental period (B). Mean and error bars are shown (n=3).

The observed degradation dynamics could be adjusted to non-linear regression and 3-parameter sigmoidal decay curves showed best results. Parameters and regression coefficients for both experimental data sets are shown in table 5.1.1. Negative controls obtained by the use of autoclaved water showed no degradation along the entire experimental period (data not shown).

	Santillana	Valmayor
a	3,82	16,45
b	-1,19	-1,32
x_0	8,70	11,85
R^2	0,89	0,99

Table 5.1.1. Parameters and regression coefficients obtained by fitting observed biodegradation of microcystins to a sigmoidal decay curve described as $f(x) = a/(1+\exp(-(x-x_0)/b))$

In the Valmayor experiments the extract contained considerable amounts of MC-RR, -YR and -LR. For all three cases, observed degradation was similar (fig. 5.1.2), lag phases of equal duration being present. Also, once degradation sets in, disappearance rates are similar for all three chemical species. Slight variations might be attributed to a limited number of data points along the degradation curve and to different initial concentration. In Santillana reservoir, the bloom extract added was clearly dominated by MC-LR, so degradation of different chemical species of the toxin could not be studied.

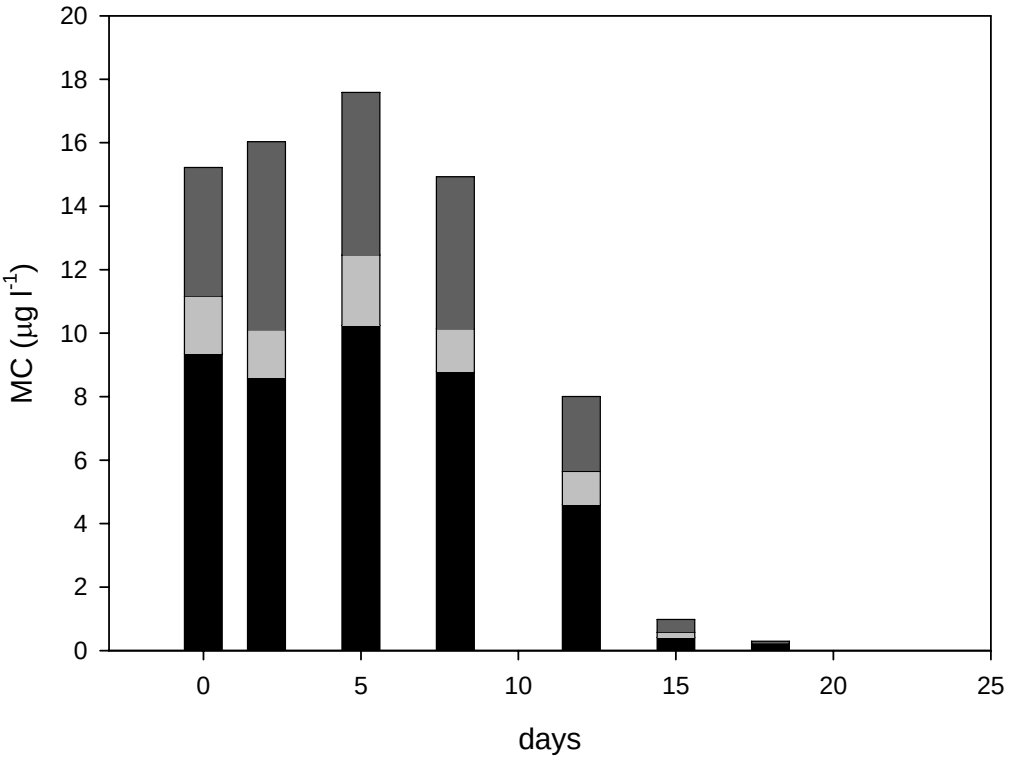


Figure 5.1.2. Biodegradation of the microcystin variants MC-RR (black bar), MC-YR (light grey bar) and MC-LR (dark grey bar) in the Valmayor microcosm experiment

Concerning bacterial abundance in the microcosms (fig. 5.1.1), the shape of growth curves in both experiments were similar. Initially quick growth is observed, largest population being achieved at days 4 and 5 respectively. Afterwards population size begins to decrease, this loss in bacterial numbers being attributed to C-limiting

conditions. Thus, main MC degradation in both cases is not occurring during the initial growth phase, but when the bacterial population is already declining.

The attempt to isolate MC degrading bacteria did not succeed. After five enrichment steps in MC supplemented MSM, aliquots from the samples in which degradation had been confirmed were transferred to solid agar plates. From these plates, we isolated 220 single colonies, which were tested for MC biodegradation capability. None of the colonies tested were able to degrade MC-LR in MSM during incubation times of 20 days. Also, in none of these colonies the gene *mlrA* could be detected by PCR analysis. Also, samples from the biodegradation experiment in the field and the final enrichment step in MSM were tested for *mlrA*, results being negative in all cases. The amplification of *mlrA* by the PCR method under our experimental conditions was confirmed as *mlrA* was very clearly detected in *Sphingomonas* ACM-3962 (photo 5.1.1.).

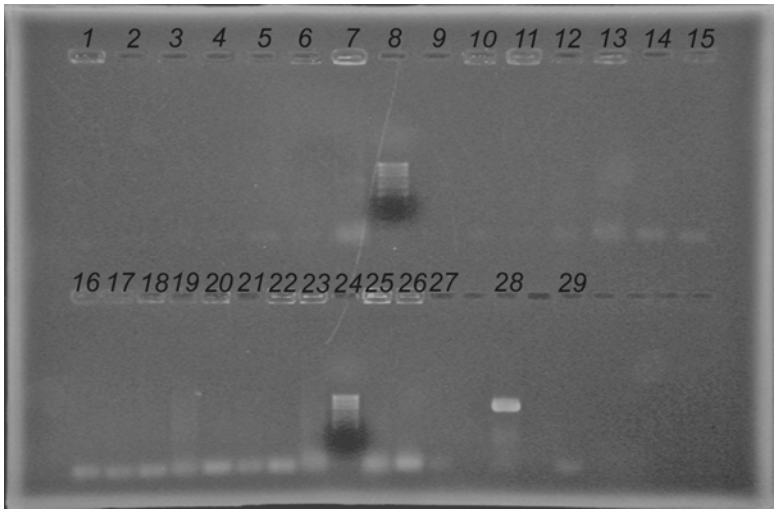


Photo 5.1.1. Example for PCR screen of samples from the MSM enrichment steps and of Sphingomonas ACM 3962 for the presence of the mlrA gene, performed as described by Saito et al., 2003. Lane 8 and 24: 100 bp; lane 1-7, 9-22 and 25-27: samples from enrichments steps in MSM, lane 28: Sphingomonas ACM 3962, lane 29: negative control.

5.1.5. Discussion

The present work firstly aimed to observe MC biodegradation under conditions that simulated naturally occurring scenarios, as laboratory experiments with purified toxin will only offer limited information regarding biodegradation in the field. Therefore, microcosm experiments were conducted at two reservoirs and different crude

MC extracts were used. We observed efficient degradation of MC in both cases. During main degradation episodes, 13 and 19% of MC in the samples was consumed each day in Valmayor and Santillana samples respectively. Thus, complete degradation can be expected to occur in less than a week once biodegradation is taking place. The degradation pattern of the three MC variants tested did not vary significantly. Similar results have been observed for example by Ishii et al. (2004) and they are of interest because different chemical species of microcystins pose different toxicological properties (e.g.: Gupta et al., 2003).

The obtained results, very rapid disappearance of MC once biodegradation sets in, are similar to degradation observed by different authors under laboratory conditions with complex bacterial communities or isolated strains (e.g.: Bourne et al., 1996; Ishii et al., 2004; Valeria et al., 2006). In contrast with some of these studies and also with Christoffersen et al. (2002), who performed their studies in microcosms, very important lag phases were observed in both of our studied systems. In Santillana reservoir, initial lag phase was shorter, but a two-step decline was observed.

Two-phase degradation has been suggested to occur due to serial degradation by different bacterial populations. For example, a first population would use MC as source of energy and carbon, afterwards a second population would be co-metabolizing the toxin (Jones and Orr,. 1994). This hypothesis was established on experimental data dealing with very high initial MC concentration. In our studies, concentration is much lower, but high amounts of other organic compounds are available. These compounds could also be promoting a serial degradation, MC being consumed as energy, carbon or nitrogen sources only when other available sources are used up.

In this way, it should be pointed out, that MC degradation in our studies does not correlate with bacterial growth. Instead, degradation occurs after maximum population size has been achieved and when bacterial numbers are already declining. Only the initial drop in MC concentration in Santillana reservoir, where the samples had been spiked with crude extracts from cyanobacteria, green algae and diatoms, is concurrent with periods of growing bacterial populations. Christoffersen et al. (2002) performed diverse studies concerning MC biodegradation and generally observed absence of lag phases and close relationship between DOC and MC consumption, thus interpreting that

the presence of labile carbon pool accelerates MC degradation. This is contradictory with our findings, as main bacterial growth is occurring before MC is consumed.

We hypothesize that initial growth is possibly sustained by other cell compounds. During this period, MC degradation, if taking place at all, might be related to co-metabolism. Competitive exclusion and lack of other labile organic compounds would afterwards reinforce the presence of actively MC degrading bacteria. A closer look at data from Christoffersen et al. (2002) shows that during field experiments, when an extract from a cyanobacterial community dominated by several species of *Microcystis* (98% of total biovolume) was used instead of a highly toxic laboratory culture, biodegradation was restrained during around 24 hours. Also, in their laboratory studies, an initial 24h-lag phase is only observed in the case where highest amounts of DOC are added to the biodegradation sample. During these 24 hours, on the other side, main bacterial growth was occurring. Our findings suggesting limited MC biodegradation during main bacterial development based on other compounds could explain these data. Considering this dependence of MC degradation on other organic carbon sources, Park et al. (2001) found that MC degradation was four times faster in an organic nutrient-free medium than in medium containing organic nutrients, thus suggesting that presence of competing substrates indeed affected biodegradation negatively. A possible explanation for this phenomenon would be the broad substrate specificity of the hydrolytic enzymes as described by Kato et al., 2007.

Further, the findings of Kato et al. (2007) indicate that the degradation capabilities of the strain tested were not induced by the degradation substrates, as cell extracts were used. Therefore, the lag phases we observed in our studies, and possibly expect at the field, should not be explained by the activation of particular metabolic pathways. Instead, presence of competing substrates or the need for competitive selection of MC degrading bacteria could be important. In the field, during bloom episodes, MC are expected to be liberated only in small amounts and to be accompanied by high concentration of other labile carbon sources. According to our results, an immediate degradation may not always be expected. This immediacy may be depending on the fact that other sources could be chosen first and – in contrast to laboratory or mesocosm experiments – continuously replaced.

The serial exposure of bacteria to MC in MSM allowed to draw some more conclusions. First of all, it is confirmed that bacteria are able to use MC as only carbon, nitrogen and energy source. Also, this biological degradation resulted in a loss of toxicity in terms of the PPA assay. These data are consequent with those from Ho et al. (2007 b), who confirmed that biodegradation resulted in a loss of toxicity as measured by both protein phosphatase 2A inhibition assay and cell-based cytotoxicity assays.

Unfortunately, the main goal of these enrichment steps, namely the isolation of MC degrading strains, was not fulfilled. The bacteria responsible for degradation, or at least for the initial steps in MC degradation, seem not to be easily cultivated on the solid media tested. Also, the attempt to detect *mlrA* in these isolated colonies failed. More surprisingly, *mlrA* was not detected neither in the biodegradation experiments in the field nor in the enrichment steps. The reasons for this can not be clearly identified. Still, in an active bacterial community growing with MC as sole carbon, energy and nitrogen source, if *mlrA* is responsible for MC degradation, it should be so strongly represented as to be detected by the PCR assay. Ho et al. (2006) were able to detect the gene even in samples from biofilms which removed MC, a sample that can be expected to be far more complex than ours.

This allows suggesting that the biodegradation observed in our study must be mediated by somehow modified *mlrA* genes or even by other gene clusters different from those proposed by Bourne et al. (2001). This is in accordance with Edwards et al. (2008), who found novel biodegradation intermediates in experiments performed with Scottish water samples. Unfortunately, as the isolation of MC degrading bacteria in our study failed, the characterisation of modified or alternative gene clusters can only be suggested, but not confirmed. Further work should be performed in order to evaluate the diversity of MC-degrading processes in the environment, factors influencing these processes and geographical distribution of this diversity. Such work may be considered mandatory in order to fulfil the aim of Saito et al. (2003) for probes that might allow rapid detection of MC-degrading bacteria in the environment.

5.1.6. Acknowledgement

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5.2. Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40 day study

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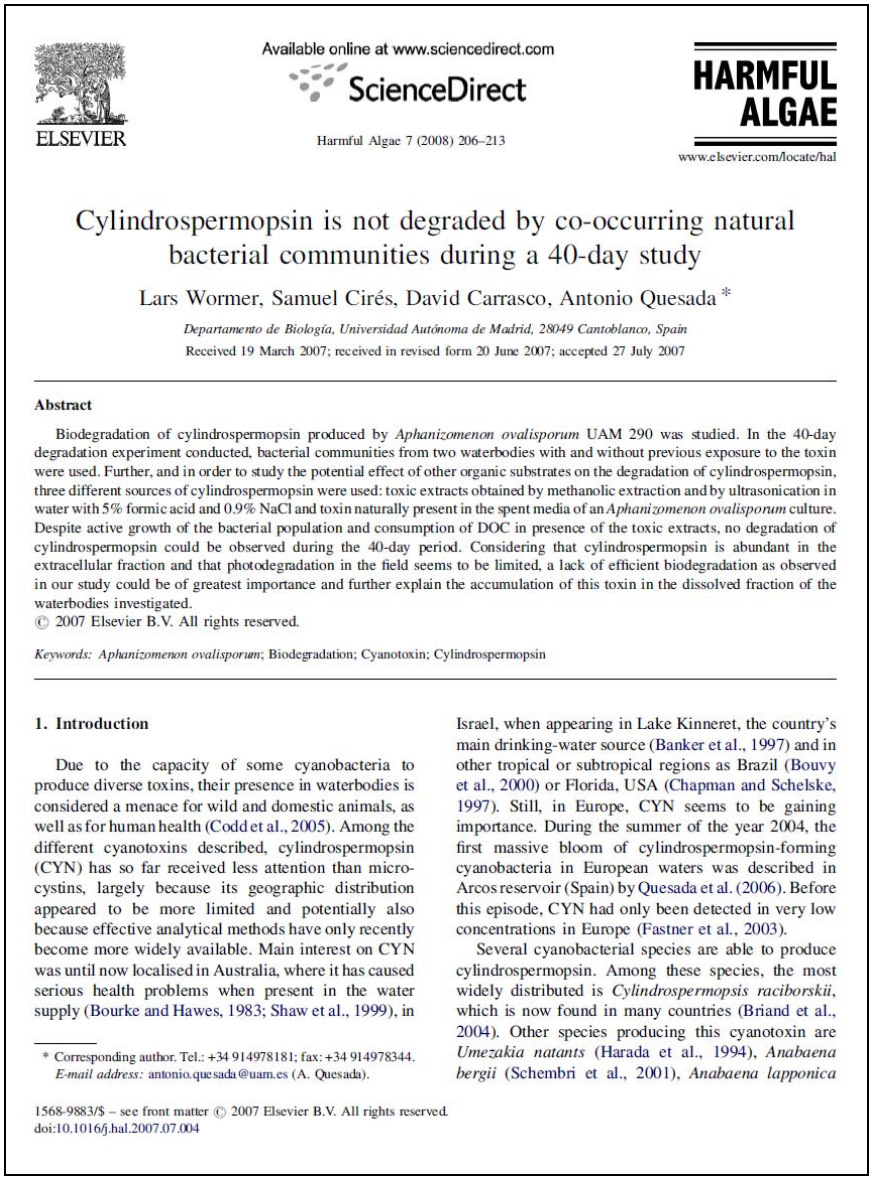

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Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study

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Abstract

Biodegradation of cylindrospermopsin produced by *Aphanizomenon ovalisporum* UAM 290 was studied. In the 40-day degradation experiment conducted, bacterial communities from two waterbodies with and without previous exposure to the toxin were used. Further, and in order to study the potential effect of other organic substrates on the degradation of cylindrospermopsin, three different sources of cylindrospermopsin were used: toxic extracts obtained by methanolic extraction and by ultrasonication in water with 5% formic acid and 0.9% NaCl and toxin naturally present in the spent media of an *Aphanizomenon ovalisporum* culture. Despite active growth of the bacterial population and consumption of DOC in presence of the toxic extracts, no degradation of cylindrospermopsin could be observed during the 40-day period. Considering that cylindrospermopsin is abundant in the extracellular fraction and that photodegradation in the field seems to be limited, a lack of efficient biodegradation as observed in our study could be of greatest importance and further explain the accumulation of this toxin in the dissolved fraction of the waterbodies investigated.

Keywords: *Aphanizomenon ovalisporum*; Biodegradation; Cyanotoxin; Cylindrospermopsin

1. Introduction

Due to the capacity of some cyanobacteria to produce diverse toxins, their presence in waterbodies is considered a menace for wild and domestic animals, as well as for human health (Codd et al., 2005). Among the different cyanotoxins described, cylindrospermopsin (CYN) has so far received less attention than microcystins, largely because its geographic distribution appeared to be more limited and potentially also because effective analytical methods have only recently become more widely available. Main interest on CYN was until now localised in Australia, where it has caused serious health problems when present in the water supply (Bourke and Hawes, 1983; Shaw et al., 1999), in Israel, when appearing in Lake Kinneret, the country's main drinking-water source (Banker et al., 1997) and in other tropical or subtropical regions as Brazil (Bouvy et al., 2000) or Florida, USA (Chapman and Schelske, 1997). Still, in Europe, CYN seems to be gaining importance. During the summer of the year 2004, the first massive bloom of cylindrospermopsin-forming cyanobacteria in European waters was described in Arcos reservoir (Spain) by Quesada et al. (2006). Before this episode, CYN had only been detected in very low concentrations in Europe (Fastner et al., 2003).

Several cyanobacterial species are able to produce cylindrospermopsin. Among these species, the most widely distributed is *Cylindrospermopsis raciborskii*, which is now found in many countries (Briand et al., 2004). Other species producing this cyanotoxin are *Umezakia natants* (Harada et al., 1994), *Anabaena bergii* (Schembri et al., 2001), *Anabaena lapponica*

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5.2.1. Abstract

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5.2.2. Introduction

Due to the capacity of some cyanobacteria to produce diverse toxins, their presence in waterbodies is considered a menace for wild and domestic animals, as well as for human health (Codd et al., 2005). Among the different cyanotoxins described, cylindrospermopsin (CYN) has so far received less attention than microcystins, largely because its geographic distribution appeared to be more limited and potentially also because effective analytical methods have only recently become more widely available. Main interest on CYN was until now localised in Australia, where it has caused serious health problems when present in the water supply (Bourke and Hawes, 1983, Shaw et al., 1999), in Israel, when appearing in Lake Kinneret, the country's main drinking-water source (Banker et al., 1997) and in other tropical or subtropical regions as Brazil (Bouvy et al., 2000) or Florida, USA (Chapman and Schelske, 1997). Still, in Europe, CYN seems to be gaining importance. During the summer of the year 2004, the first massive bloom of cylindrospermopsin-forming cyanobacteria in European waters was described in Arcos reservoir (Spain) by Quesada et al (2006). Before this episode, CYN had only been detected in very low concentrations in Europe (Fastner et al, 2003**).**

Several cyanobacterial species are able to produce cylindrospermopsin. Among these species the most widely distributed is *Cylindrospermopsis raciborskii,* which is now found in many countries (Briand et al*.,* 2004). Other species producing this cyanotoxin are *Umezakia natants* (Harada et al., 1994), *Anabaena bergii* (Schembri et al., 2001), *Raphidiopsis curvata* (Li et al., 2001) and *Aphanizomenon ovalisporum* (Banker et al*.,* 1997, Shaw et al., 1999, Quesada et al., 2006). Recently, Preussel et al. (2006) also described a strain of *Aphanizomenon flos-aquae* as CYN-producer.

Cylindrospermopsin is an alkaloid which acts as a potent protein synthesis inhibitor. The main target of CYN in vertebrates seems to be the liver, but other organs such as the thymus, kidney, adrenal glands, lungs, intestinal tract and heart may also be affected. Besides other episodes, CYN has been implicated as cause of hepatoenteritis on Palm Island, Australia, affecting 148 people (Bourke and Hawes, 1983; Bourke et al., 1986). Further, assays have shown CYN-induced genotoxicity (Humpage et al., 2000, 2005; Shen et al., 2002) and evidence for carcinogenity (Falconer and Humpage, 2001). Due to high solubility in water and apparent membrane permeability, important

amounts of the toxin can be expected to occur in a soluble state. This has been confirmed by Norris et al. (2001) for a *Cylindrospermopsis raciborskii* culture. Shaw et al (1999) suggested that in the case of *Aphanizomenon ovalisporum* the release of toxin into water could be even higher.

Effective chemical degradation of cylindrospermopsin has been achieved with chlorination, provided the concentration of other organic matter is low (Senogles et al., 2000) and by photocatalytic degradation with titanium oxide and UV irradiation (Senogles et al., 2001). In spite of the availability of effective water-treatment techniques the presence of CYN in water constitutes a risk if people are exposed to untreated water via direct water contact e.g. through recreation or occupational use or if affected waters are not adequately treated.

In-situ degradation of the toxin can be expected to occur mainly due to photo- and biodegradation. Photodegradation of high concentrations of cylindrospermopsin is effective when a toxic cyanobacterial extract with high concentrations of plant pigments is directly exposed to sunlight (Chiswell et al., 1999). Pure CYN or CYN added to water containing natural, and thus lower, amounts of plant pigments is degraded poorly (Chiswell et al., 1999). No data on biological degradation of CYN have been reported in the literature.

Our aim in this work was to fill this gap by establishing the extent of biological degradation of CYN by indigenous co-occurring bacteria. Previous exposure to a compound is commonly suggested to be substantial for an optimal biodegradation (Sivonen and Jones, 1999), and we expected this to be true also for cylindrospermopsin.

Therefore, our study included investigating the importance of previous exposure of the bacterial community to the toxin by using bacterial communities from two different waterbodies. Further, the composition of the CYN source used in the experiment could be thought to influence biological degradation by processes like co-metabolism or substrate competition. To evaluate these processes, we designed three degradation scenarios, in which biodegradation would occur in presence of organic substrates of different quantity and quality.

5.2.3. Materials and Methods

Two waterbodies from the Madrid region were used in our studies. Santillana reservoir is located 40 km north of Madrid, Spain. This reservoir (maximum capacity = 91.2 hm^3, surface area = 1044 ha, maximum depth = 26 m) supplies drinking water for the city of Madrid and surrounding areas. Santillana reservoir is an eutrophic system with recurrent cyanobacterial blooms during the summer stage; these blooms being usually dominated by diverse species of *Microcystis* genera.

The pond in Parque Juan Carlos I (Madrid, Spain) (JC I) is a shallow system of artificial channels located in one of the biggest green areas of Madrid. During the summer of the year 2005, the phytoplanktonic community was constantly dominated by cyanobacteria. This pond is used for recreation during summer, although no bathing is allowed.

The pond in JC I was sampled at three different time-points during summer and autumn 2005. Samples were GF/F-filtered and both filter and filtrate stored at -20 ºC for further analysis. The seston retained on the filters was extracted with 0.9% saline solution containing 5% formic acid by pulse-pestle ultrasonication (three 30-s pulses). CYN in the filtrate was concentrated by lyophilization and resuspended in distilled water. HPLC analysis was performed on a Waters Alliance 2695 HPLC system with a 996 PDA detector equipped with a Waters Spherisorb 5µm ODS2 column. The filtrate was collected and analysed for CYN by the HPLC-PDA system according to the protocol described by Törökné et al. (2004). The presence of CYN in the field samples was verified by its UV spectrum and its retention time and quantified by comparison to injected standards. In some cases, the presence of CYN was confirmed via the addition of internal standard.

Buoyant cyanobacterial genera were determined by microscopy on fresh unfiltered samples. The sample was left undisturbed for 24 h, after which floating and sedimented cyanobacteria were collected from the surface and bottom and identified at the microscope. For *Aphanizomenon ovalisporum* quantification in samples from JC I on October 27th, an Olympus BH-2 microscope was used. Five millilitres water samples from the sampling point were filtered through Anodisc 25 mm filters in triplicate. The biovolume of each filament was estimated by considering it as an ideal cylinder and

measuring relevant geometric dimensions of each filament. Thirty random fields of view per filter were selected and total biovolume estimated by counting and calculating the sum of all filaments present in the fields and by taking field and filtration areas into account. Finally, total biovolume was calculated as the average of the three filters, and referred as mm^3 of *Aphanizomenon ovalisporum* per m^3 of water.

Aphanizomenon ovalisporum was isolated from Parque JC I after the sampling on September 5th. For this purpose, individual filaments were transferred to 24-well plates and grown in $BG11_0$ (Rippka et al., 1979). Successfully isolated strains were transferred into 50 ml Erlenmeyer flasks.

Five strains were isolated from JC I during the month of September and identified as *Aphanizomenon ovalisporum* (photo 5.2.1). These strains were named from UAM 287 to UAM 291 consecutively. The production of CYN by these organisms was confirmed (data not shown) and strain UAM 290 was selected as CYN source for the degradation experiment.

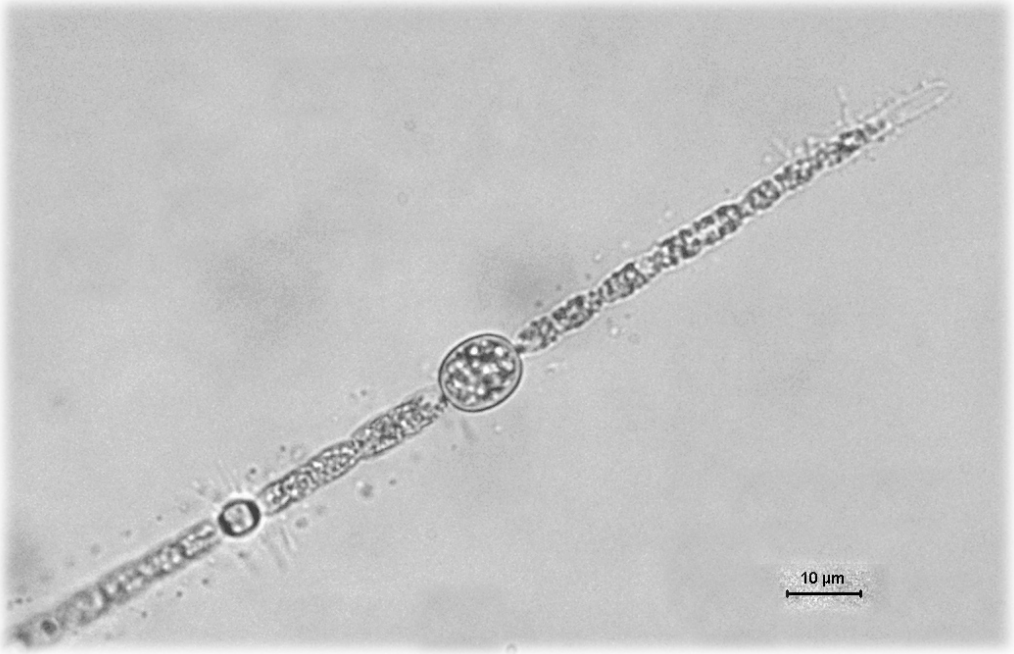

Figure 5.2.1: Aphanizomenon ovalisporum from Parque de Juan Carlos I (Madrid, Spain)

For the degradation experiments, GF/C filtered water and bacteria were obtained on October 27th from two sources which differed in their previous exposure to CYN: the same artificial pond in Parque de JC I from which the toxic organism had been isolated and from Santillana reservoir (Madrid, Spain), a reservoir with an extensive previous

history of toxic cyanobacteria (e.g. Carrasco et al., 2006). Diverse studies have been carried out in this reservoir in the past, results indicating that the only potential CYN-producer observed over the last years is *Aphanizomenon flos-aquae*, which recently, and so far, only in one case has been described to produce CYN (Preussel et al., 2006). Fortnightly sampling during summer of the years 2004 and 2005 indicated absence of CYN in the reservoir.

Aphanizomenon ovalisporum UAM 290 was grown and harvested when achieving the stationary phase. Cells were separated from the spent media by centrifugation. Both supernatant and pellet were further used as CYN source in the experiment.

The experimental design aimed to allow possible biodegradation to take place in scenarios where the toxin was accompanied by different substrates which could enhance or limit degradation processes. Therefore, different CYN sources were used. The medium in which *Aphanizomenon ovalisporum* had been grown, once centrifuged, was freeze-dried. It was expected that this source would contain water-soluble organic lysates as well as inorganic nutrients present in the original medium. Cell-bound CYN was extracted in two different ways: extraction into distilled water by pulse-pestle ultrasonication as described above and extraction into pure methanol after sonication. Aqueous extraction would free the highly soluble fraction, while the methanol-extracted fraction would also include more hydrophobic compounds.

These three sources of CYN (dissolved fraction, aqueous CYN extract with ultrasonication and methanol-extracted) were added to bacterial communities with previous exposure to CYN to a final concentration of about 40 $\mu g l^{-1}$ for the dissolved fraction and 100 $\mu g l^{-1}$ in the cell-bound fractions. The toxic extract obtained by ultrasonication was also added to bacteria without previous exposure to CYN and to sterilized water and materials to a final concentration around 100$\mu g l^{-1}$. A control case was obtained by not adding any CYN source to water from JC I. The experiments were incubated in the dark at a constant temperature of 28 ºC. Samples were taken after 0, 12 and 24 hours and after 2, 4, 7, 14 and 40 days. HPLC analysis of CYN, dissolved organic carbon (DOC) measurements by TOC and bacterial countings by flow cytometry were performed.

HPLC analysis of the samples was carried out by freeze-drying of the samples and resuspension in distilled water. CYN concentration was determined as described above for natural samples.

Samples for flow cytometry were fixed with formaldehide to a final concentration of 2% (Lebaron et al., 1998) and stored in darkness at 4º C. Staining with SYTO 9 obtained from Molecular Probes (LIVE/DEAD *Bac*Light Bacterial Viability Kit) was performed before analysis on a Beckman Coulter Cytomics FC 500 MPL cytometer. Calibration for quantification was performed by addition of beads in known concentration to the analysed samples.

DOC concentration was measured with a high-temperature catalytic oxidation method in a Shimadzu TOC analyzer (Model V CSH/CSN) equipped with a platinized-quartz catalyst for high sensitivity analysis. Samples were purged for 20 min to eliminate remains of dissolved inorganic carbon (DIC). Three to five injections were analysed for each sample and blank (Milli-Q water). Standardization of the instrument was done with potassium hydrogen phthalate (4-points calibration curve).

5.2.4. Results

Aphanizomenon ovalisporum was present at the artificial lake in Parque de Juan Carlos I during the whole summer season, although the dominant species was *Microcystis aeruginosa*. Beside *Aphanizomenon ovalisporum,* no other potential CYN-producers were found during the sampling period. Cylindrospermopsin was detected on all three samplings (Table 5.2.1). While sestonic CYN concentration remained quite constant during the whole sampling period, with concentrations around 3 $\mu g l^{-1}$, the extracellular presence of CYN constantly increased. This led to final concentrations of 7.83 $\mu g l^{-1}$ in the dissolved fraction, three times more than the co-occurring sestonic concentration.

Date	Sestonic CYN (μgl^{-1})	Dissolved CYN (μgl^{-1})	Ratio dissolved/sestonic fraction
10/08/2005	2.66	2.93	1.10
05/09/2005	3.70	6.54	1.76
27/10/2005	2.63	7.83	2.97

Table 5.2.1. CYN concentrations in the pond in Parque de Juan Carlos I (Madrid, Spain) during summer 2005

During the degradation experiment, net degradation was not observed in any of the cases (fig. 5.2.1). Only smaller variations were observed at some concrete timepoints, but concentrations always remained above 90% of the initial concentration and showing no temporal pattern.

Concerning the importance of other possible substrates for the degradation of CYN, no differences were observed between the diverse CYN sources used (fig. 5.2.1). Whether using the methanolic extract, the extract obtained by ultrasonication into water or the CYN naturally present in the spent media, no degradation was observed.

Also the origin of the inocula used, and their previous exposure to the toxin, does not seem to be of importance (fig. 5.2.1). Bacteria from JC I used for the degradation experiment had been concurrent with an estimated biovolume of 72.3 mm^3 *Aphanizomenon ovalisporum* per m^3 of water and more than 10 μgl^{-1} total CYN concentration. This bacterial community, with a proven previous exposure to *Aphanizomenon ovalisporum* and CYN, showed to be just as incapable to degrade the toxin as the bacteria from Santillana reservoir, where no CYN has been detected so far. In the controls conducted with sterilized water, as expected, no degradation was observed.

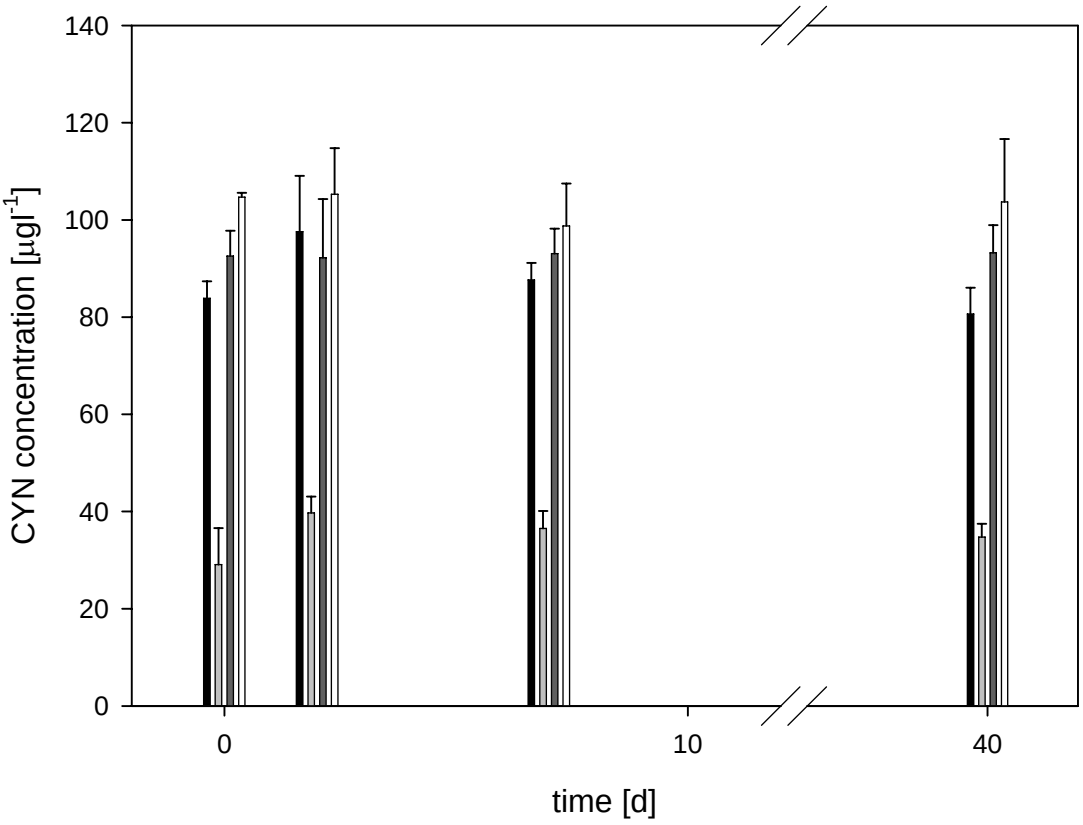

***Figure 5.2.1:** Variation of CYN concentration during the 40-day experimental period. Bacterial communities with previous natural exposure to the toxin (Parque JC I) are exposed to CYN obtained by methanol-extraction (black bar), by recovery of CYN present in spent medium (light grey bar) and by aqueous extraction with ultrasonication (dark grey bar). Bacterial communities without previous contact with CYN (water from Santillana reservoir) are also exposed to CYN obtained by aqueous extraction with ultrasonication (white bar). Values are the mean of three replicates, error bars representing standard deviation.*

Concerning bacterial growth, positive growth in the experimental flasks was observed in all of the experiments (fig. 5.2.2). Bacterial growth was much more pronounced in the experiments in which the methanolic extract or the soluble fraction were added to the substrate, and their decrease during the later phase of the experiment was also highest. The extract obtained by ultrasonication seems to allow only very small growth rates.

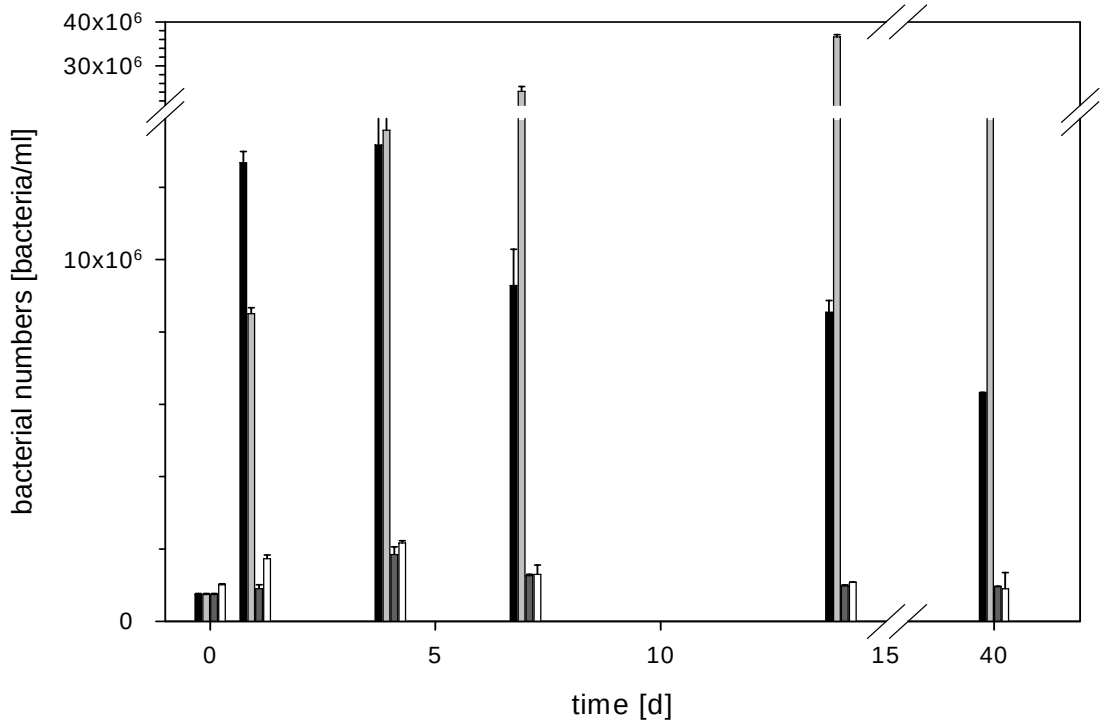

Figure 5.2.2: Bacterial numbers during the CYN biodegradation experiment over a 40 day period. Bacteria from Parque JC I exposed to extracts obtained by methanolic extraction (black bars), by recovery of CYN from spent media (light grey bars) or by ultrasonication (dark grey bar) and bacteria from Santillana reservoir exposed to CYN obtained by ultrasonication (white bar). Values are the mean of three replicates, error bars representing standard deviation.

The growth of the bacterial population in the experiment is reflected by the consumption of available DOC. In all of the non-sterilized experiments, a significant decrease in the concentration of DOC is observed (table 5.2.1), final concentration being lower than 50% of the initial concentration. The lowest remaining percentage was obtained when the toxic extract was added to water and bacteria from Santillana reservoir, only about 10% of the initial DOC persisted after the complete experimental period.

Origin of bacterial community	Parque JC I				Santillana reservoir
CYN source	No CYN added	CYN in spent medium	Methanol-extracted	Extracted by ultrasonication	Extracted by ultrasonication
DOC remaining	21,9 ± 0,3%	34,9 ± 1,1%	40,5 ± 0,8%	46,3 ± 4,2%	10,7 ± 2,3%

Table 5.2.2. DOC remaining after the 40d experimental period expressed as percentage of the initial concentration present at day 40. Media and standard deviation (n=3) are represented.

5.2.5. Discussion

The present work shows how an active microbial community, with previous exposure to moderate amounts of CYN is not able to degrade this cyanotoxin even under laboratory conditions that should be optimal. Neither previous exposure to the toxin nor the characteristics of the CYN source added seem of importance as no degradation is observed over a total period of 40 days in none of the cases. Meanwhile, the bacterial population shows strong growth, and other compounds – expressed as DOC – are effectively being consumed. Beside other considerations, these results also allow to infer that CYN does not seem to interfere in the activity of the bacterial community, although some taxa could have been affected by this toxin. This remains open to further investigation. The different bacterial growth rates observed are attributed to the different extracts used and thus to the different amount and quality of substrates available in each one.

The obtained results show that biodegradation of CYN under the conditions given is not taking place. This, together with natural accumulation observed in the field, indicates biological degradation of this toxin under natural conditions to be very slow or limited to specific conditions not tested here. Considering that in situ biodegradation is

commonly accepted as the main path to limit the presence of other cyanotoxins –as for example microcystins – in the environment (Sivonen and Jones, 1999), this possible lack of degradation represents an additional difficulty in CYN risk management.

Meanwhile, photodegradation has shown to be effective in laboratory studies with addition of rich algal extracts, but is limited when CYN is added to natural water samples with lower levels of pigments (Chiswell et al., 1999), the half-life of the toxin increasing dramatically in this case. Further, in the field, turbidity or other factors affecting the depth of the photic zone will clearly limit the effectiveness of photodegradation.

These difficulties in the degradation of CYN make the toxin susceptible to accumulation in the system. Furthermore – in contrast to microcystins – cylindrospermopsin is extraordinarily soluble in water and tends to be massively liberated from the cells to the surrounding media. Enhanced liberation, high solubility and limited biodegradation should therefore be responsible for the accumulation of substantial concentrations of this toxin in the dissolved state, as described by Chiswell et al. (1999) and Shaw et al. (1999) in the field or Norris et al. (2001) in a laboratory culture. In the present study, an increase of the concentration of the dissolved fraction over time was indeed also observed in the field. In October 75% of total CYN was extracellular. Thus, net accumulation, probably related to the limited biodegradation of the toxin, seems to be a widespread phenomenon.

If additionally it is considered that *Cylindrospermopsis raciborskii* seems to be behaving as an invasive species (Briand et al., 2004) and *Aphanizomenon ovalisporum* could be following a similar path, the risk associated with CYN should be taken seriously. Not only the production of this toxin in more temperate systems could be enhanced, also an ineffective degradation could lead to increasing accumulation of cylindrospermopsin in our waters, especially with senescent blooms and the associated liberation of the toxin, which could persist long after the producing cells have disappeared. Further questions to be answered would also concern the fate of cylindrospermopsin once accumulation in the dissolved fraction has taken place.

5.2.6. Conclusions

(i) Biodegradation of cylindrospermospin by an active microbial community does not take place during a 40-day laboratory study.

(ii) Neither the composition of the cylindrospermopsin source used, nor previous exposure of the bacterial population to cylindrospermopsin, seems to influence this lack of biodegradation.

(iii) The accumulation of cylindrospermopsin in the dissolved fraction of waterbodies, and the risks associated, might be related to this inefficient biodegradation.

5.2.7. Acknowledgements

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5.3. Natural photodegradation of the cyanobacterial toxins microcystin and cylindrospermopsin

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5.3.1. Abstract

Microcystins (MC) and cylindrospermopsin (CYN) are potent toxins produced by diverse cyanobacterial genera found in waterbodies throughout the world. In the present study, we assessed the photodegradation of MC and CYN by different radiation bands of the natural solar spectrum (PAR, UV-A and UV-B) and along the water column. Photodegradation of CYN seemed to be highly dependant on UV-A, and was very low in the field. This fact could be one of the reasons explaining the high extracellular CYN concentration found in diverse waterbodies. Microcystin photodegradation was higher, all three radiation bands (PAR, UV-A and UV-B) being responsible for its degradation, although PAR and UV-A revealed more important because of their higher natural irradiance. As MC-degradation rates for the different radiation bands could be established and specific attenuation profiles of radiation estimated, we were able to perform basic modelling of MC photodegradation along the water column. Running the proposed model yielded significant degradation potential in shallow systems or thin mixed layers. To further complete the understanding of these processes, photosensitizer-dependent degradation rates for each radiation band may be incorporated into the model. Generally speaking, MC photodegradation shows the benefit of its immediate response, but may lack efficiency in deeper systems.

5.3.2 Introduction

Cyanobacterial massive blooms have become a usual phenomenon in waterbodies throughout the world (Codd, 2000), this dominance being of concern because of the ability of some cyanobacteria to produce diverse toxins (Carmichael, 1992). The most common of these toxins, the microcystins (MC), are cyclic heptapeptides containing two variable amino acids and the unusual aromatic amino acid ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid). They act as potent hepatotoxins, inhibiting serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990). Tumour-promoting activity (Falconer, 1991), gastroenteritic and hepatic diseases, and irritant reactions have been linked to the presence of MC (Dawson, 1998 and references therein). Microcystins are accumulated by the producers as intracellular metabolites and liberated to the surrounding medium mainly by cell death and lysis (Rohrlack and Hyenstrand, 2007). Concentrations of dissolved MC in environmental samples are generally one or more orders of magnitude lower than those in seston (Poon et al., 2001; Oh et al., 2001). This fact is explained by limited liberation, but also by means of dilution and *in situ* degradation. Biodegradation has been described to efficiently take place in a variety of systems (e.g.: Christoffersen et al., 2002; Edwards et al., 2008), but also the effect of solar radiation has been considered as a potential source of degradation. For example, experiments by Tsuji et al. (1994) showed photodegradation of MC to non-toxic products. This degradation occurred under natural sunlight irradiation, but demanded high concentrations of photosensitizing pigments to be added. Later, Welker and Steinberg (2000) confirmed such findings by demonstrating that degradation rates of microcystins depended linearly on the absorbance of tested waters, no degradation taking place in Milli-Q water. Also, in this work, the authors were able to show that MC photodegradation in a range of 5 to 55 µg l^{-1} was following first-order kinetics, degradation rate being unaffected by initial toxin concentration.

Cylindrospermopsin (CYN) is a small toxic alkaloid produced by diverse genera of cyanobacteria. Toxicity of CYN seems to be affecting a variety of organs due to its ability to penetrate into a wide range of cells (Chong, 2002). Protein inhibition (Froscio et al., 2003) seems to be responsible for most of the acute damage by CYN and some of its metabolites. Futher, DNA damage (Shen et al., 2007) and carcinogenic activity has

been suggested (Falconer and Humpage, 2001). In the past, CYN has been considered typical for tropical or subtropical regions, as Australia (Shaw et al., 1999), Israel (Banker et al., 1997), Florida (Chapman and Schelske, 1997) or Brazil (Bouvy et al., 2000). Lately its presence has been expanding to more tempered regions as for example Spain (Quesada et al., 2006), Germany (Fastner et al., 2007), Poland (Kokocinski et al., 2009) or Czech Republic (Bláhová et al., 2009). In contrast to MC, CYN seems to be actively liberated by the producing organism, Mihali et al. (2008) even described the possible existence of CYN transporters. This enhanced liberation, together with limited biodegradation seems to be partly responsible for the high extracellular concentrations observed in field samples (Smith et al., 2008, Wörmer et al., 2008). Photodegradation of cylindrospermopsins has only received limited attention. Senogles et al. (2001) demonstrated the efficiency of titanium dioxide as photocatalyst. Chiswell et al. (1999) studied stability of cylindrospermospins under variable pH, temperature and light. Their results demonstrated the photodegradation potential of sunlight when photosensitizers are available.

Considering the risk of human exposure to cyanobacterial toxins, *in situ* degradation is important both because of the possibility of direct exposure to the toxin, for example in recreational waters, and because optimal water treatment facilities are not always available. Thus, a realistic evaluation of potential photodegradation in the field and along the water column is required. To prospect these potentialities, a series of experiments were conducted in a freshwater reservoir, these experiments focused on the photodegradation of MC and CYN at reasonable concentrations, under natural conditions and over an ecologically relevant period.

In addition, two factors were considered to be decisive in understanding the relevance of photodegradation in the field. On the one side, and for the first time, the contribution of the diverse bands of solar radiation: PAR (400-700 nm), UV-A (320 – 400 nm) and UV-B (280-320 nm) was studied, thus allowing to identify the wavelengths which mainly account for the degradation of the toxins. On the other side, the importance of the water column as filter for radiation was evaluated by estimating photodegradation in samples deployed at several depths along the watercolumn and by performing a complete survey of attenuation coefficients for 48 waterbodies around Spain. The correct understanding of photodegradation by the different radiation spectra

and of the vertical extinction of radiation in the water column can allow a realistic estimation of the photodegradation in natural ecosystems. This estimation may be crucial for an evaluation of the risks associated to cyanobacteria and their toxins.

5.3.3 Materials and Methods

Microcystins and cylindrospermopsins were obtained from exponentially growing laboratory cultures of *Microcystis aeruginosa* UAM 247 and *Aphanizomenon ovalisporum* UAM 287 respecively. After harvesting of *Microcystis*, cells were extracted by 10 minutes sonication in methanol 90% (v/v). The microcystin extract contained the chemical species MC-LR, MC- RR and MC-YR. Extraction of CYN was performed by pulse-pestle ultrasonication in saline solution (0.9%) containing 5% formic acid. After centrifugation, the supernatants of three consecutive extractions were pooled, vacuum-dried and resuspended in distilled water.

The filter-sterilized (0.2 μm pore size) toxic extracts were then added to Whirl-Pak bags (Nasco Ltd.) filled up with 200 ml GF/F filtered and autoclaved (121ºC, 20 min) Valmayor reservoir water (Madrid, Spain) to a final concentration of 18.32 μg l^{-1} total MC and 33.6 μg l^{-1} CYN. Afterwards, these bags were attached to racks deployed offshore at Valmayor reservoir on October 18th (photo 5.3.1). Microcystin producing cyanobacterial blooms are most often detected in Spanish waterbodies during the months of July, September and October (Carrasco et al., 2006), therefore October was considered a month suitable for bloom decay, toxin liberation and thus exposure of the toxins to photodegradation. The racks were incubated for 22 days under five different conditions: Three racks were placed floating on the Valmayor reservoir surface, and kept in contact with the surrounding water in order to avoid temperature shifts in the samples. To establish exposure to different solar radiation bands, one of the racks was left uncovered (exposure to PAR + UV-A + UV-B) while the other two were covered with Mylar (exposure to PAR + UV-A) and OP3 (exposure to PAR) sheets respectively. OP3 is opaque to wavelengths below 400 nm, while in the case of Mylar opacity is to wavelengths below 320 nm. In addition to their opacity to certain radiation bands, both Mylar and OP3 and also the sampling bags used are not completely transparent to the remaining radiation spectrum. Average transmittance values for PAR and UVR and transmittance values for irradiances at 305 nm and 380 nm have been considered when

establishing correlations between degradation of toxins and radiation received in the samples.

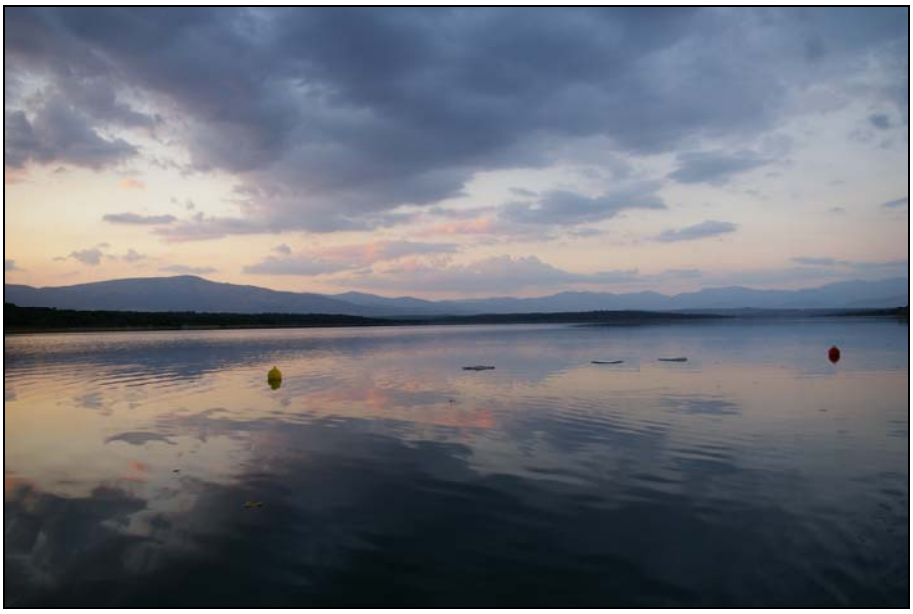

Photo 5.3.1: Experimental setup deployed at Valmayor reservoir

A fourth rack was placed at one meter depth below water surface and the last one was deployed at four meter depth. None of these two were covered by any additional artificial UVR absorbing material. By deploying these racks, we intended to describe how photodegradation is affected by attenuation of radiation along the water column, thus providing a realistic view of these processes under natural conditions. Negative controls were obtained by placing bags inside completely opaque containers at 4 meter depth

Sample bags were withdrawn in triplicate after 2, 5, 12 and 22 days. The samples were kept at 4ºC, transported to the lab in less than two hours and afterwards frozen and stored for toxin quantification. Additionally, subsurface samples from Valmayor reservoir for dissolved organic carbon (DOC) measurement and chlorophyll *a* (chl a) quantification were obtained. Chlorophyll *a* was extracted in methanol and measured spectrophotometrically (Marker et al., 1980) on a Shimadzu Multi-Spec 1501. DOC concentration was measured with a high-temperature catalytic oxidation method in a Shimadzu TOC analyzer (Model V CSH/CSN) equipped with a platinized-quartz catalyst for high sensitivity analysis. Samples were purged for 20 min to eliminate remains of dissolved inorganic carbon (DIC). Three to five injections were analysed for

each sample and blank (Milli-Q water). Standardization of the instrument was done with potassium hydrogen phthalate (4-points calibration curve).

Samples for MC analysis were concentrated by solid phase extraction on C18 cartridges and analysed on a Waters Alliance 2695 HPLC system with a 996 PDA detector equipped with a Purospher STAR RP-18 column (250 x 4.6 mm, 5 μm) following the method proposed by Lawton et al. (1994). Additionally, toxicity of the samples due to MC and/or possible degradation products, was evaluated by protein phosphatase inhibition assay (PPA) by the use of a MicroCystest kit (ZEU Inmunotec, Zaragoza, Spain). Samples for CYN analysis were stored frozen (-20ºC) until analysis on a Waters Acquity ultra-performance™ liquid chromatograph system (Milford, MA, USA) coupled to a 3200 Qtrap hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Chromatographic separation was performed using an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) preceded by a C18 guard column both supplied by Waters (Waters Corp., Milford, MA, USA). Separation was performed with a binary mobile phase at a flow rate of 0.5 mL/min. The separation conditions were as follows: solvent (A) acetonitrile with 0.1% formic acid; solvent (B) water with 0.1% formic acid. The isocratic elution was: 0-0.1 min, 0% A; 0.1-5 min, 0-100% A; 5-5.2 min, 100% A; 5.2-6.0 min, return to initial conditions; 6.0-7.0 min, equilibration of the column. Acquisition was performed in the selected reaction monitoring (SRM) mode and the protonated molecular ion was chosen as the precursor ion.

Considering irradiation measurements, daily UVR values and irradiances at 305 nm, 310 nm, 324 nm and 380 nm at local solar noon were obtained by Matlab subroutines from the OMUVB data products generated using satellite measurements from the OMI (Ozone Monitoring Instrument). Global radiation measurements for the closest meteorological station were provided by Agencia Estatal de Meteorología, measured in a wavelength range from 305 to 2800 nm every second and integrated for the obtention of daily values. Based on these measurements, we applied the factors proposed by González and Calbó (2002) and Howell et al. (1983) to convert global radiation to PAR energy.

Once radiation over the water surface had been quantified, its behaviour along the water column was considered of interest. Attenuation of PAR in the upper 4 m of

the water column in Valmayor reservoir was measured at morning, noon and evening during the day of the experimental deployment. These measurements were performed with a cosine-corrected 2π LI-COR LI-190 air light sensor measuring simultaneously with a cosine corrected 2 π LI-COR LI-192 underwater light sensor with a vertical resolution of 0.2 m, and logged in a Li-1000 (LI-COR). Values were computed as percent of transmission for each depth, considering radiation just below the water surface as 100%.

In the case of UVR, attenuation coefficient (K_d) was estimated by absorption coefficients (α) and/or DOC concentrations using the equations in Morris et al. (1995). Absorption coefficients for wavelengths from 200 to 750 nm were obtained by diode array spectrophotometry (Shimadzu Multi-Spec 1501) in 1 or 10 cm pathway cuvettes, spectral resolution being 1 nm. For UVR absorption coefficients, quartz cuvettes were used. We here applied the relationships proposed for 305 and 380 nm and considered them representative for UV-B and UV-A respectively Additionally to the experimental data from Valmayor reservoir, water samples from 48 waterbodies around Spain were processed within 24 hours of sampling.

Modelling of potential photodegradation at different depths along the watercolumn was performed on Stella software v.8 (High Performance Systems Inc.).

5.3.4 Results

Weather conditions were stable and sunny over the whole experimental period, even though a very significant drop in PAR is observed during the fifth and sixth day of exposure (fig. 5.3.1 A). In the central part of the experimental period, a few episodes of increased cloudiness and thus a decrease of UVR received were observed (fig. 5.3.1 B). Accumulated UVR and PAR measured during the whole period was 30.4 MJ m^{-2} and 132.5 MJ m^{-2}, respectively. To evaluate the importance of UV-A and UV-B separately, irradiance at specific wavelengths (305, 310, 324 and 380 nm) was taken into account (fig. 5.3.1 B). Irradiance for these wavelengths follows similar trends, and average ratio of irradiances at 305 and 380 nm is 0.0399.

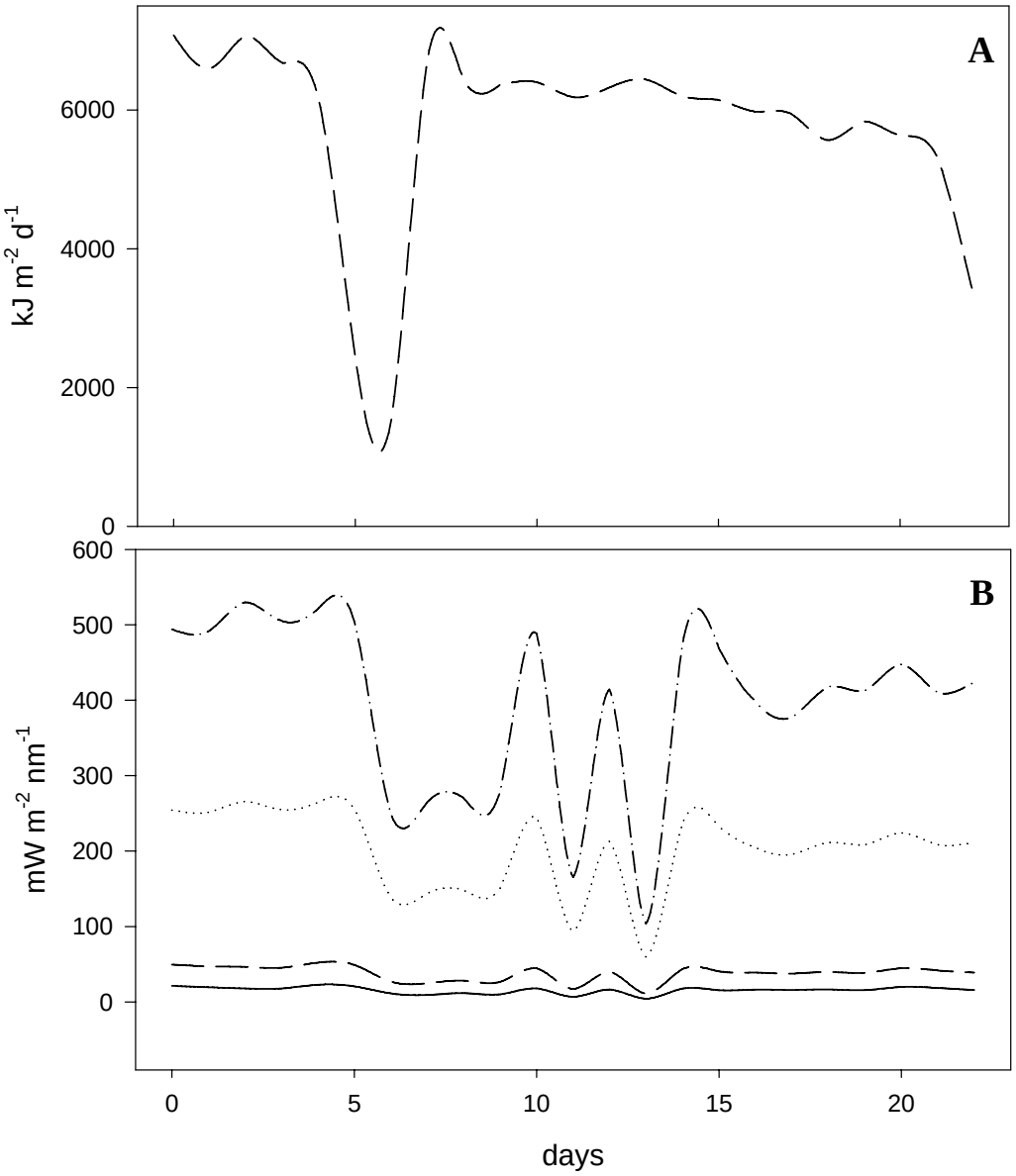

Figure 5.3.1: a) PAR and b) irradiance at local solar noon for wavelengths 305 nm (solid), 310 nm (dashed), 324 nm (dotted), and 380 nm (dot-dash) at Valmayor reservoir during the sampling period

Besides quantifying incoming radiation, it was important to evaluate the transmission characteristics of radiation along the watercolumn. The studied reservoir was characterised by high initial chlorophyll *a* concentration (33.1 µg l^{-1} in subsurface samples). After 12 days, the concentration rapidly dropped to 12.4 µg l^{-1} chl *a*. After this initial drop, concentration continued decreasing, being only 6.6 µg l^{-1} after the 22 day experimental period. DOC concentration from subsurface samples in the reservoir was relatively stable during the initial 12 days, increasing afterwards. The lowest concentration (10 mg l^{-1}) was detected on day 5, while average DOC concentration was 15.3 mg l^{-1}. Vertical profiles of PAR transmittance measured at the day of experimental

deployment along the upper 4 m of the water column were similar at morning, noon and evening and showed good fit to an exponential decay curve (R^2 = 0.996; 0.989 and 0.972 respectively). The average attenuation coefficient for downwelling PAR ($K_{d\ PAR}$) was 1.014 ± 0.253 m^{-1}. Considering the radiation reaching depths at which the racks were deployed, at 1 m an average attenuation of 59.1% was observed, at 4 m PAR attenuation was 97.8%.

UV radiation attenuation along the water column could not be obtained in the field, instead, we used equations proposed in Morris et al. (1995) to estimate K_d considering absorption coefficients (α) and DOC concentration (table 1). Attenuation coefficients obtained from α for Valmayor reservoir are 9.05 and 28.0 for UV-A (380 mm) and UV-B (305 nm), respectively. When estimating K_d with DOC, values clearly increase. Considering both K_d estimations, 99% attenuation of UV-A (380 nm) is predicted to occur between 0.24 and 0.51 m, while attenuation of 99% of UV-B (305 nm) is predicted to occur between 0.10 and 0.16 m (table 5.3.1).

	K_d (m^{-1})	$Z_{1\%}$ (m)
K_d predicted by DOC		
380 nm	19.61	0.235
305 nm	44.29	0.104
K_d predicted by absorption coefficient		
380 nm	9.05	0.509
305 nm	28.0	0.164

Table 5.3.1. Estimation of the vertical attenuation coefficient K_d as a function of DOC (average DOC concentration: 15.28 mg l^{-1}) or absorption coefficients at 380 nm (UV-A) and 305 nm (UV-B) as described by Morris et al. (1995). K_d for Valmayor reservoir is predicted and used to establish penetration of 1% incoming UV radiation ($Z_{1\%}$)

Considering MC photodegradation at the water surface, samples directly exposed to the full solar radiation spectrum showed that 78.7% ± 2.15% of initial MC concentration was degraded after 22 days. Both exposure to PAR + UV-A and only PAR resulted in a more limited degradation of MC than in the samples exposed to the complete solar spectrum (fig. 5.3.2). Final concentrations showed a degradation of 64.7% ± 2.12% of the initial concentration when the samples were exposed to PAR and UV-A and 29.2% ± 1.18% when samples were exposed only to PAR. By comparing degradation rates over time in the different cases, importance of the different radiation bands could be evaluated. It was observed that up to 35.2% of degradation in the uncovered samples could be attributed to PAR, and 64.8% to UVR. UV-A contributed more to the photodegradation of MC (46.6%) than UV-B radiation (18.2%)

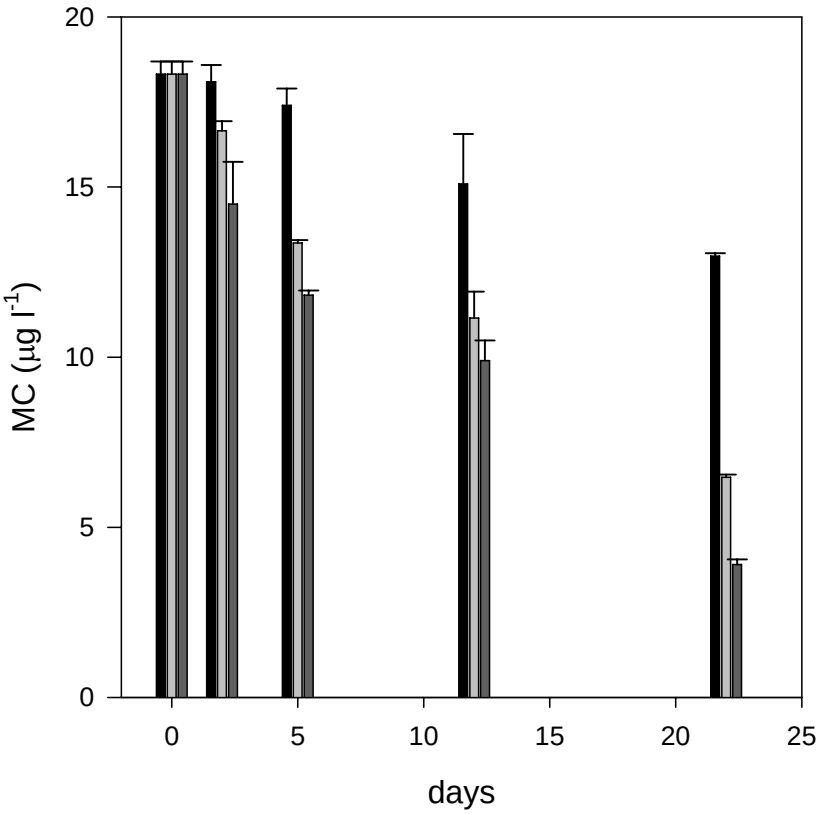

Figure 5.3.2: Photodegradation of microcystins (MC) in samples placed at the water surface and exposed to solar PAR (black bars), PAR + UV-A (light grey bars) and full solar spectrum (dark grey bars) along the 22 days experimental period in Valmayor reservoir.

Beside degradation over time, it is interesting to obtain degradation rates considering the radiation energy received in the samples. When applying linear models

to the degradation of MC in uncovered samples and plotting residual MC concentration against the accumulated radiation energy for the whole spectrum (PAR + UVR), a degradation rate of 0.137 ng MC l^{-1} (kJ m^{-2}) $^{-1}$ was obtained (R^2= 0.935). In addition to this global degradation rate, the UV-opaque (OP3 covered) sample allows us to estimate a specific degradation rate due to PAR. The degradation rate obtained was 0.064 ng MC l^{-1} (kJ m^{-2}) $^{-1}$, the linear model showing very good fitting (R^2 = 0.968). In the case of specific degradation rates for UV-A and UV-B, estimations are not possible because no sample set was exposed only to one of these radiation bands, but a stepwise approach comparing the different degradation cases and the radiation bands involved showed good results. We considered 305 and 380 nm as representative wavelengths for UV-B and UV-A, and expressed degradation rates as related to irradiance at local noon. Obtained degradation rates were 2.727 ng MC l^{-1} (mW m^{-2} nm^{-1})$^{-1}$ for irradiance at 380 nm and 8.902 ng MC l^{-1} (mW m^{-2} nm^{-1})$^{-1}$ for 305 nm. UV-B degradation rate thus is about three times greater than UV-A, but this high degradation potential only yielded limited photodegradation in the field, as the higher degradation rate is counterbalanced by the lower radiation received in the UV-B range.

Concerning the question of vertical attenuation of solar radiation and its effect on photodegradation, MC concentration was studied at three different depths (fig. 5.3.3). As shown above, samples on the water surface showed a decrease of MC concentration of nearly 80% and a degradation rate of 0.137 ng MC l^{-1} (kJ m^{-2})$^{-1}$. At 1 and 4 meter depth, degradation could be observed to lesser extent, and only 24.4% ± 3.70% and 9.9% ± 3.43% of initial MC had disappeared at the end of the experimental period. It should be noted though, that while degradation in the samples on the water surface was close to constant during the experimental period, in the underwater samples degradation seems to be accelerated between days 5 and 12.

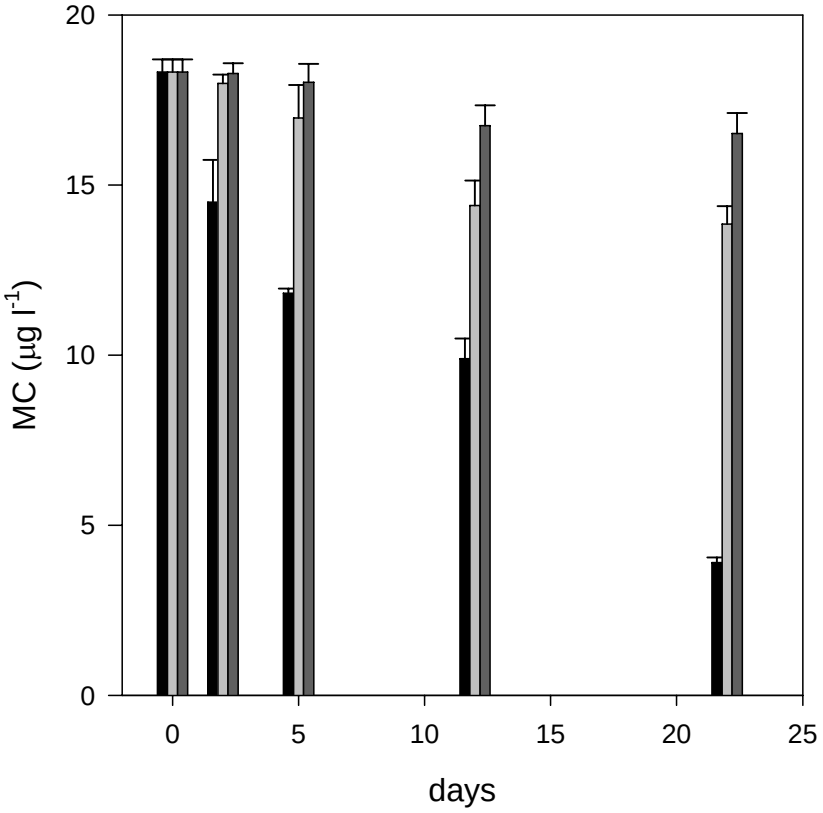

Figure 5.3.3: Photodegradation of MC in samples exposed at the water surface (black bars), at 1m depth (light grey bars) and at 4m depth (dark grey bar) without any additional artificial protection from UVR

When studying the photodegradation of toxic compounds, it is important that the degradation observed by the analytical method chosen is accompanied by a real decrease of its toxicity. Therefore, besides confirming the photodegradation of MC by HPLC, evolution of toxicity of the samples due to MC and possible photodegradation products was also evaluated by the use of a protein phosphatase assay. When relating concentrations determined by HPLC and the phosphatase assay, a strong correlation was observed ($R^2 = 0.977$). In other words, the decrease in the concentration measured by HPLC directly related to a loss in toxicity.

Degradation of CYN was very low under the experimental conditions given. When exposed to full sunlight at the water surface, only 27.3% ± 5.83% of initial CYN

was degraded along the 22 day experimental period. When protecting these samples from UV radiation, degradation was even lower. PAR+UV-A exposed samples showed a toxin decrease of 20.6% ±9.50%, while in samples exposed only to PAR, no degradation was observed. Finally, experimental setups placed along the watercolumn also revealed very limited photodegradation of CYN. At one meter depth, decrease in toxin concentration was 10.8% ± 4.35%, samples at 4 m depth showed no variation in CYN content.

In the case of microcystins, where important degradation potential was observed, we afterwards focused on establishing a simplified model which could forecast potential photodegradation at different depths along a theoretical water column. This model is based on the specific MC degradation rates calculated for the different radiation spectra and is fed with K_d values which may be measured or estimated through α or DOC values. Further, the model is driven in function of irradiance values and initial MC concentration supplied. Mixing parameters, depth of the watercolumn and depth intervals for which predictions are made may also be modified.

This model is applied to representative attenuation coefficients obtained from 48 Spanish waterbodies which had been sampled in October 2008. A frequency distribution of the attenuation coefficients for PAR, UV-A (380 nm) and UV-B (305 nm) estimated for these waterbodies following Morris et al. (1995) are shown in fig. 5.3.4. For each distribution, first and third quartiles and median were obtained (fig. 5.3.4) and used to recreate low- medium- and high- K_d degradation scenarios with the model proposed above.

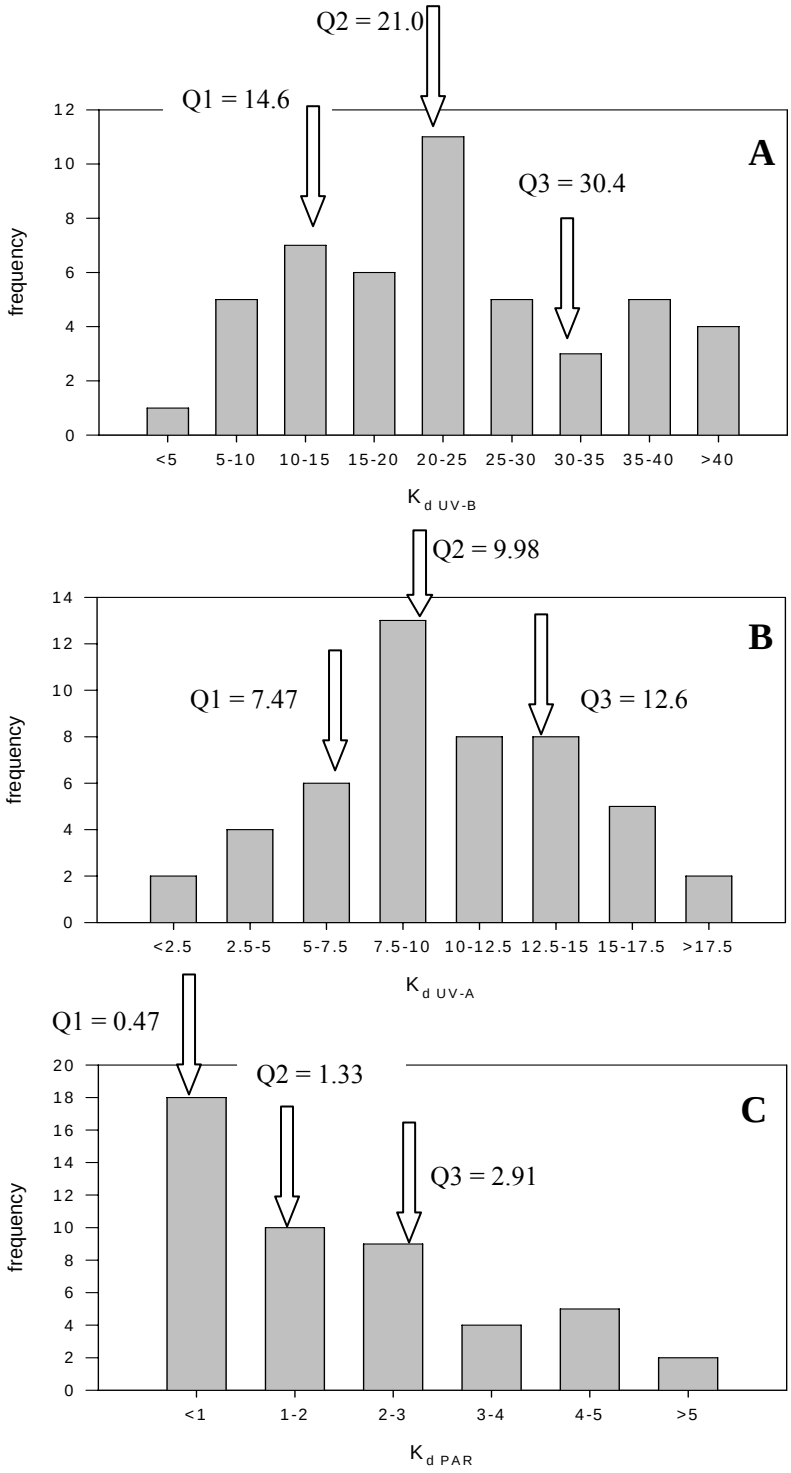

Figure 5.3.4: Frequency distribution of attenuation coefficients (K_d) for 380nm, 305nm and PAR in 48 Spanish waterbodies estimated through absorption coefficients (Morris et al., 1995). For each distribution, first (Q1) and third (Q3) quartiles, as well as median (Q2) values are shown.

The use of non-mixed watercolumns in the model (fig. 5.3.5), although they are not representing conditions as expected in the field, allows to show the importance of

vertical attenuation of radiation. Increasing attenuation translates in a considerable narrowing of the layer in which photodegradation is active. A sharp transition from relevant degradation rates to strongly limited photodegradation is explained by attenuation of UVR, while the slower photodegradation along greater depths is related to PAR mediated degradation.

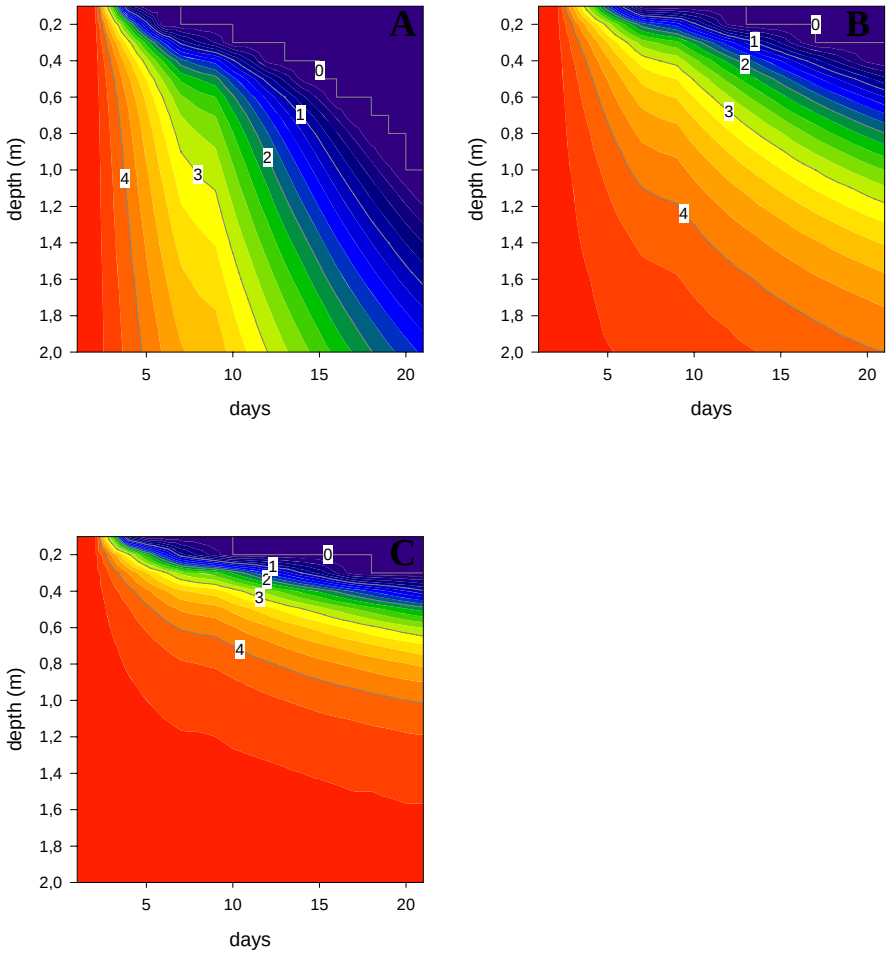

Figure 5.3.5: Prediction of photodegradation of MC over 22 days at 2m depth under low (A), medium (B), and high (C) K_d scenarios. Initial MC concentration modelled is 4.5 µg l^{-1}

A model including daily mixing of the watercolumn shows higher similarity with naturally occurring processes. Table 5.3.2 resumes the half-life for MC estimated by our model considering the three K_d scenarios described above in mixed watercolumns of 2, 6 and 10 m depth. These depths were chosen to represent shallow systems (2 m), but also deeper, stratified systems in which mixed layer is being build up (6 m) or completely established (10 m). We may observe that in shallow systems, half-life remains low, even with highest attenuation values, according to our estimations, half-life of MC may be estimated between one or two weeks. An increase of the depth of the mixed layer translates in strongly increased half-life values.

	Low K_d	Medium K_d	High K_d
2 m	6.3	9.3	11.5
6 m	13.5	23.8	31.6
10 m	21.5	39	52

Table 5.3.2: Half-life (days) of microcystins under variable K_d conditions and in a mixed watercolumn of 2, 6 and 10 m depth.

Taking this strong variability of degradation into account, the performance of a sensitivity analysis for our model was estimated convenient. Sensitivity (S) gives us an idea of the effect that a change in the values of one parameter has on certain variables of interest. In our case, we chose half-life of 5 µg l^{-1} MC along a 6 m-watercolumn as the variable of interest. Studied parameters (table 5.3.3) were depth and attenuation coefficients and degradation rates of PAR, UV-A and UV-B. Values for medium- K_d scenarios were chosen as starting values and relative variations of 10 and 50% were applied. Highest impact on half-life was caused by a modification of the depth of the mixed layer. Both K_d and degradation rates for PAR and UV-A also had significant impact on the system behaviour, while in the case of UV-B very large variations are needed for even small responses in the overall degradation.

		10% variation	50% variation
UV-B	K_d	0	0.01
	Degradation rate	0.05	0.05
UV-A	K_d	0.12	0.23
	Degradation rate	0.43	0.36
PAR	K_d	0.45	0.48
	Degradation rate	0.49	0.41
Depth		0.81	0.95

Table 5.3.3. Sensitivity (S) analysis for half-life of microcystins in a 6 m mixed water column under medium K_d conditions

5.3.5. Discussion

The experimental data from *in situ* photodegradation at Valmayor reservoir offer an improved understanding of the importance of such degradation in the field, and of the environmental factors limiting its activity. Photodegradation of cylindrospermopsin by sunlight in natural waters was very low. After a 22 day exposure to full sunlight, 72.7% of initial concentration remained unaltered. UV-A may be expected to mainly contribute to such degradation, as CYN concentration did not decrease in samples exposed only to PAR but decreased in similar amounts when exposed to PAR+UV-A and PAR+UV-A+UV-B. Also, the protective effect of the watercolumn was confirmed, as degradation was very limited at one meter depth and was completely arrested at four meter.

It may be of interest to compare these results with the findings from Chiswell et al. (1999). These authors demonstrated that CYN could be degraded by sunlight, and that the presence of photosensitizers was crucial to allow such degradation. Still, most of these experiments were performed under extraordinary high CYN and photosensitizer

concentrations. When testing photodegradation of low CYN concentrations in natural waters, they observed much slower degradation, establishing a half-life of between 11 and 15 days. If having a closer look on their results, it is surprising to observe that in their experiments, CYN concentration remains almost stable along one week, then suffers a rapid decline along a short time and again stabilizes afterwards. Unfortunately, no information concerning received radiation along the experimental period is given by the authors, an information that could be especially interesting given that the experiments, to our knowledge, were performed in Brisbane (Australia), where high incoming UV-R may be expected.

We suggest that CYN photodegradation may only be taking place when high amounts of photosensitizers are available and under very high irradiation, especially in the UV-A range. Together with higher excretion of CYN by producing organisms (Saker and Griffiths, 2000, Preussel, 2008) and limited biodegradation (Smith et al., 2008; Wörmer et al., 2008), this fact may be contributing to the high amounts of CYN observed in the extracellular fraction (e.g.: Shaw et al., 1999; Rücker et al. 2007).

Microcystins though, under the same experimental conditions, were confirmed to be degraded in a significantly greater amount (fig. 5.3.2 and 5.3.3). The degradation rate of MC in relation to accumulated UVR and PAR radiation is 0.137 ng MC l^{-1} (kJ m^{-2})$^{-1}$. If we only considered UVR, as it is done for example in the studies of Welker and Steinberg (1999), a rate of 0.883 ng MC l^{-1}(kJ m^{-2})$^{-1}$ is obtained (R^2 = 0.965). This rate is lower than the values obtained in the mentioned study for MC-RR in presence of high amounts of fulvic acid (3.6 ng MC l^{-1} (kJ m^{-2})$^{-1}$). These differences are consistent with their findings concerning the importance of fulvic acids on indirect photolysis, which clearly accelerates the photodegradation processes. In Welker and Steinberg (2000), it was stated that degradation rates in fulvic acid spiked waters was 3 or 4 times higher than in natural waters with equal absorbance. In the present work and in order to keep experimental conditions as close as possible to field conditions, we consciously used filtered water from Valmayor reservoir, with naturally present DOC, and only added the DOC which was obtained along with the MC and CYN sources during extraction processes. It should also be noted that, as photosensitizer sources were not replenished, the long experimental periods may be exhausting these photosensitizers, thus explaining the slightly slower degradations at the later days.

Concerning possible toxicity of degradation products, we can confirm that alteration of MC directly relates to a loss in toxicity in terms of PPA. This could indicate that photodegradation is actually affecting the ADDA aminoacid, which has been found indispensable for the toxicity of microcystins and nodularins. Such findings are consequent with photodegradation pathways proposed by Liu et al. (2003) or Antoniou et al. (2008).

The use of different radiation filters allowed us to identify the radiation responsible for degradation in the field, a characterization which so far had not been performed. Also specific MC-degradation rates for the different radiation bands could be established. Highest degradation rates in terms of received radiation are observed for UV-B. But even if its potential is high, in the samples exposed to the complete radiation range, MC degradation is carried out mainly by UV-A (46.6%) and to a lesser extent by PAR (35.2%). The lower potential degradation rates of UV-A and PAR seem to be counterbalanced by the great amount of radiation in these longer wavelengths (fig. 1b). It is important to point out that PAR in this surface samples contributes to degradation in a considerable amount, being about twice as important as UV-B and nearly as important as UV-A. These data are similar to those obtained by Graneli et al (1996) for photodegradation of dissolved organic carbon. Also, Vähätalo et al. (2000) quantified the impact of the different radiation bands on the degradation of dissolved organic carbon in humic lakes and observed that UV-B contributed only 9%, while UV-A was the main degradation agent (68%), followed by PAR (23%).

When studying photodegradation in the field, and especially in deep systems as the reservoirs dealt with in the present work, the study of the extinction of radiation along the water column, and thus the limitation of photodegradation is essential. Diverse authors have studied extinction of UV in water bodies of different kind and DOC has been widely identified as the most suitable predictor (Scully and Lean, 1994; Morris et al., 1995; Granéli et al., 1996; Huovinen et al., 2003). From the different models suggested to estimate K_d for UV-B and UV-A for a given DOC concentration, we chose to use the equations proposed in Morris et al. (1995). The results obtained are consequent with the data found in diverse studies where 1% UVR penetrated deeper than one meter only in the clearest systems (e.g.: Bukaveckas and Robbins-Forbes, 2000; Huovinen et al., 2003).

PAR radiation, on the other hand, shows much greater transmittance across the water column. Measuring extinction of PAR in the water column of Valmayor reservoir, we observe that at one meter depth, transmitted radiation is still high, an average of 40.9% of the radiation received at the surface. Also at 4 m depth between 1.5 and 5% of PAR radiation is still recorded, thus at both depths PAR radiation can be expected to be the only radiation contributing to photodegradation.

The experimental data concerning specific degradation rates and extinction of the different radiation bands along the water column were used in the model proposed and could help establishing potential photodegradation in the field. Under the conditions recorded in Valmayor reservoir during the experimental period, both at one and four meter depth, the degradation predicted by our model (data not shown) is lower than the degradation observed during the photodegradation experiment. This fact can be related to a possible underestimation of the radiation reaching this depth as a consequence of the fact that the PAR extinction profile, highly dependent on DOC and chl *a* concentration, was performed under conditions less favourable to transmission than those found during the later experimental period.

Attenuation coefficients obtained from the survey of 48 waterbodies around Spain offered a complete image of representative K_d values. Obtained values are quite high, but it should be noted that samples are littoral, offshore values may be expected to be lower (e.g.: Hayakawa and Sugiyama, 2008). Also, the K_d estimations are based only on DOC absorption, not considering particulate organic matter. This decision is grounded on the findings of studies like those performed by Scully and Lean (1994) or Morris et al. (1995) which found attenuation to depend essentially on dissolved organic matter. Still, studies like those conducted by Belzile et al. (2002) in Lake Biwa (Japan) showed that particulate organic matter can significantly contribute to attenuation of both UVR and PAR. Lake Biwa is characterised by relatively low DOC values, a fact that could be partially explaining the higher importance of particle scattering and absorption. Still, attenuation coefficients in this study have to be considered carefully, keeping in mind that we may be overestimating penetration and thus photodegradation when only considering absorption by DOC, ignoring the effect of particles (e.g. scattering).

Quartiles and median values of the frequency distributions were calculated (fig. 5.3.4) and used in our model to evaluate potential photodegradation in scenarios of low,

medium and high attenuation of radiation and in mixed layers of 2, 6 and 10 m (table 5.3.2). We observe a sharp transition from the shallow system, in which photodegradation plays a major role, to depths in which it is very limited. Translated to the field, this could be interpreted as efficient photodegradation to be expected only in shallow systems or during stages in which the mixed layer of deep, stratified systems is still thin. The importance of depth is also confirmed by the sensitivity analysis (table 5.3.3) performed, which was clearly dominated by this parameter. Sensitivity analysis also confirms that UV-B does not play an important role in photodegradation in the field. Attenuation and degradation rate of PAR and UV-A are shown to strongly influence the outcome of our model, PAR being apparently more important than UV-A. At this point, it should be considered that the decrease of dissolved organic constituents will always benefit radiation transmission, but at the same time will translate in lower degradation rates, as photodegradation of microcystins is occurring indirectly. The equilibrium between these two aspects will therefore define viability of photodegradation in the field and the impact of variable absorbance of natural waters. Welker and Steinberg (2000) were able to establish a linear relation between the UV-related degradation rate constant and α_{350} in a range from 1.9 to 7.4 m^{-1}. If comparing this relation with the relations expressed in Morris et al. (1995) in which K_d is estimated by α, we observed that the impact on K_d seems to be higher than de impact on degradation rates. Therefore, decrease in organic components could, at least in a certain range, benefit photodegradation of MC. Variation of degradation rates in the diverse radiation bands due to variations in absorbance, as those described by Welker and Steinberg (2000) for UVR, could be incorporated in future photodegradation models in order to guarantee even higher confidence.

The obtained results may contribute to a better overall understanding of the importance and the limiting factors regarding photodegradation of MC and CYN in the field. Photodegradation of CYN, seems to be very limited under natural conditions, and to strongly depend on UV-A radiation. Further work has to be carried out in order to elucidate the fate of CYN in the environment. In the case of microcystins, photodegradation might be more important and PAR, even if having smaller degradation potential, can be expected to play an important role along the water column due to its deeper penetration. This degradation potential may have been underestimated as studies traditionally have been carried out over shorter time periods and focusing on

UV radiation. Still, photodegradation may be expected to play an important role in the fate of microcystins only under some concrete situations, for example in shallow systems or narrow mixed layers in deep, stratified systems, with high residence times and during lag phases of concurring biodegradation.

5.3.6. Acknowledgements

Lars Wörmer and Samuel Cirés were supported by a FPU grant from Ministerio de Ciencia e Innovación (MICIIN, Spain). We thank Manuel Bañón (Agencia Estatal de Meteorología) for the global radiation data. Thanks also to Dr. Margarita Yela for providing the Matlab subroutines and to Dr. Ana Justel for her very valuable help in the application of these subroutines. We would also like to thank Dr. Isabel Reche and the Department of Ecology from Universidad de Granada. Special thanks to Dr. Rubén Sommaruga for very helpful advice and corrections. Our acknowledgement to Canal de Isabel II for allowing sampling and deployment of the experimental setup at Valmayor reservoir.

6. Sediments as a sink for cyanobacterial toxins

Sedimentation has been often identified as a very important stage in the annual cycle of cyanobacteria. We suspect that, if cyanobacterial toxins remain inside the organism, or are adsorbed to settling organic or inorganic matter, sedimentation may also play a crucial role in the distribution of these toxins. Therefore, we tried to establish microcystin sedimentation rates by deploying sedimentation traps in three reservoirs.

Sorption of cyanobacterial toxins to organic or inorganic matter will be important because it might increase sedimentation rates of the toxin, but also because it might be responsible for the sequestration of the toxin in the sediments, lowering its availability. Therefore we studied the sorption behaviour of both microcystins and cylindrospermopsin. The findings related to sorption of cyanobacterial toxins will also guide us in the selection of appropiate extraction methods for these compounds from the sediment.

6.1. Importance of natural sedimentation in the fate of microcystins in *Microcystis* dominated cyanobacterial communities

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6.1.1. Abstract.

Sedimentation processes were studied in three reservoirs located in Central Spain in which the cyanobacterial community was dominated by the genus *Microcystis*. Microcystins (MC) were detected in the sediment traps deployed in all reservoirs. In Santillana reservoir, microcystins were detected in sediment traps even though they could not be detected in the pelagial samples. In the other reservoirs studied, microcystins sedimentation rates during the bloom period ranged from 0.43 to 2.53 mg MC m^{-2} d^{-1}. In Valmayor reservoir, due to higher resolution sampling along the watercolumn, we could estimate that during the *Microcystis* dominated bloom, around 4.5% of pelagial microcystins may be involved in sedimentation processes at any given time-point. Interestingly, this very high sedimentation of toxic biomass is not related to decaying blooms or autumnal sedimentation due to a drop in water temperature. Instead, it seems that microcystin containing colonies may be settling constantly during the bloom. Further, these settling colonies seem to maintain good cell integrity and microcystins seem not to be excreted massively. A certain loss of MC content along the vertical settling may be attributed to minor losses due to cell lysis or to variations in microcystin cell quota due to reduced production. Our results definitely identify sedimentation as a major destination for microcystins in deep systems. Further studies concerning the fate of settled microcystins and the optimization of managing strategies concerning this source of microcystins are needed.

6.1.2. Introduction

Microcystins are potent toxins produced by a diversity of cyanobacterial genera, for example *Microcystis* (Botes et al., 1982), *Oscillatoria* (*Planktothrix)* (Sivonen et al., 1990), *Anabaena* (Sivonen et al., 1992), etc. Microcystins (MC) act as potent hepatotoxins, inhibiting serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990). Tumour-promoting activity, gastroenteritic and hepatic diseases and irritant reactions have been linked to the presence of microcystins (Falconer, 2005). Microcystins are cyclic heptapeptides containing two variable amino acids and the unusual aromatic amino acid ADDA. The different occupation of the two variable positions, as well as other structural variations, provides up to eighty variants of microcystins (Sivonen and Jones, 1999; Humpage, 2008). Among these, toxic effect and chemical properties may vary (Gupta et al., 2003).

Microcystins are accumulated by the producers as intracellular metabolites, and are expected to be liberated mainly during lysis of the producing cells (e.g.: Wiedner et al., 2003; Rohrlack and Hyenstrand, 2007). This limited liberation, as well as efficient degradation *in situ*, translates in extracellular concentrations in environmental samples generally remaining one or more orders of magnitude below those of the intracellular fraction (Poon et al., 2001; Oh et al., 2001). Also, this limited liberation implicates the importance of understanding the processes to which intracellular toxin is subjected, as such understanding will provide useful information concerning correct management of the risks associated with microcystins. In this sense, we hypothesize that whole-cell sedimentation might be a key factor in the fate of toxins caged inside producing organisms. To test this hypothesis, studies were performed in the field, in three reservoirs in which the presence of the best known microcystin producer: the genus *Microcystis*, was present. We focused on the sedimentation rates of microcystins, comparing it with concurrent intra- and extracelular pelagic microcystins concentration, and aiming to understand the overall importance of these processes in the fate of the toxin. Further, it was interesting to include large, thermally stratified systems, in order to test the in-depth variation of sedimentation parameters.

The experimental design and the interpretation of the obtained results are inspired by the different studies that have dealt with annual life cycles of the genus *Microcystis*. These studies demonstrated the importance of sedimentation processes,

overwintering in the sediments and recruitment from the sediments in spring (e.g. Reynolds, 1980; Preston et al., 1980). More recent studies, as those conducted by Ihle et al. (2005) or Welker et al. (2007), have continued exploiting the close relationship between pelagic and benthic communities of *Microcystis* sp, explaining this phenomenon by deposition of the pelagic colonies. Verspagen et al. (2005) performed a complete sedimentation and recruitment study of *Microcystis* at eight sampling sites in lake Volkerak (Netherlands). The obtained results indicated, among other aspects, that sedimentation rates of *Microcystis* were highest in September and that the sedimentation flux did not differ significantly between different parts of the lake. The authors identified the sediment as a very important sink of *Microcystis* colonies, and even stated that it acts more as a sink than as a source for the genus.

Still, these data focus mainly on the potential MC producing organisms, and not on the toxin itself, and also look more on the resulting benthic communities than on the sedimentation fluxes on their own. This translates in higher uncertainty due to external factors as could be benthic growth or decay, and variation of MC cell quota through variation in MC production or losses during settling and in the sediments. We considered it necessary to asses the hypothesis of massive intracellular MC sedimentation, not by estimating it from sedimentation of potential producers or from MC content in sediments, but by actual *in situ* measurement. An important sedimentation flux of microcystins along the watercolumn, if confirmed, could have dramatic effects on ecosystem quality and management strategies.

6.1.3. Materials and Methods

Sampling was carried out in three reservoirs located in Central Spain: Cogotas reservoir (Duero river watershed) during the year 2006 and Valmayor and Santillana reservoirs (Tajo river watershed) during the year 2007. Characterisation of the studied reservoirs is shown in table 6.1.1. The sampling period started when abundant presence of cyanobacteria was notified: August 7th at Cogotas reservoir, July 25th at Santillana reservoir and August 30th at Valmayor reservoir.

	Maximum Volume (Hm^3)	Surface Area (ha)	Average depth (m)*	Maximum depth (m)	Geographic Coordinates
Santillana	91	1044	8.7	36	40º 42' 28' 'N 03º 49' 02'' W
Cogotas	58.7	394	14.9**	60**	40º 43' 15'' N 04º 41' 42'' W
Valmayor	124	755	16.4	51	40º 31' 39'' N 04º 03´ 19'' W

Table 6.1.1. Characterisation of the studied reservoirs *average depth is calculated by dividing maximum volume by maximum area ** due to massive water withdrawal, depth of the watercolumn was drastically reduced. Maximum depth observed during our studies was 15.3 m.

During the first sampling, sediment traps were deployed in the reservoirs at two depths: at the upper metalimnion and in the hipolimnion, concretely one meter above the sediment. In Cogotas reservoir massive withdrawal of water resulted in limited depth and mixed waterlayers; therefore, only one set of traps was used. The sediment traps were designed in the laboratory and constructed by SEGAINVEX (Universidad Autónoma de Madrid) and consisted of 3 cylinders of 4.4 cm internal diameter (photo 6.1.1). The traps were deployed vertically and wrapped with black tape to prevent photosynthetical growth. When sampling, supernatants in the traps were carefully poured off in order to discard buoyant plankton. After homogenization of settled matter, aliquots were taken for quantification of organic and inorganic matter content, and particle associated microcystins. Organic (OM) and inorganic matter (IM) were quantified by obtaining dry weight of aliquots after desiccation (100ºC, 24 h) and combustion (500ºC, 4h) in porcelain cups. OM was calculated as the difference between dessicated and combusted weights. Another aliquot was GF/F filtered for microcystin determination. Microcystins were extracted by sonication into methanol (90%), concentrated under vacuum and prepared for quantification by HPLC. Quantification of MC was achieved following the procedure proposed by Lawton et al. (1994). The HPLC-PDA system (Alliance, Waters) consisted of a Waters Separations Module 2695,

equipped with a Waters 996 PDA. The chromatography column was a Purospher RP 18 endcapped (5μm) 4.6 mm x 250 mm column. Chromatograms were monitored at 238 nm and toxin concentration determined by comparison to the injected standards for MC-LR, RR and YR. The loss of sediment traps on August 28th and September 11th explains the gaps in the data from Santillana reservoir.

Photo 6.1.1.:Sedimentation trap

The water column was sampled simultaneously, sampling took place at the same site of trap deployment and always between 11 and 12 am. Vertical profiles of temperature, chlorophyll *a* (chl *a*) and dissolved oxygen were obtained with an YSI 6920 multiparameter probe. Subsurface samples and samples from the depth where the traps had been deployed were taken with a 5-liter watersampler (Uwitec). In addition, during the first sampling date in Valmayor reservoir, a higher resolution study of the watercolumn was performed, sampling at five depths (0.5, 4, 7, 10 and 16m). Samples were stored at 4ºC during transport to the lab and processed within two hours. Chl *a* quantification and algal group composition was obtained by fluorometry (Moldaenke bbe Algae Analyser). Also, after GF/F filtering, sestonic MC content was quantified by HPLC. Dissolved MC was quantified in filtered samples after concentration on C18 cartridges (Mega Bond Elut, Varian Inc.).

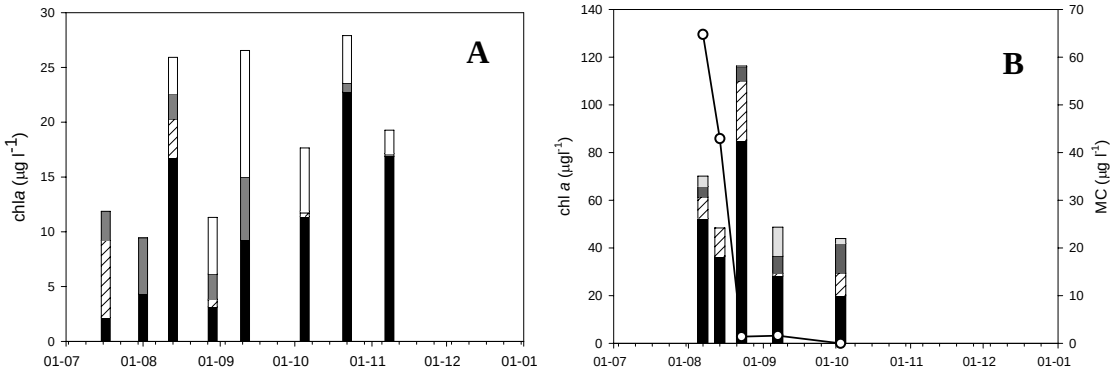
Additionally, on September 25th, daily movement of the phytoplanktonic community along the watercolumn was studied in Valmayor reservoir. The vertical distribution of microcystins at morning, noon and evening was studied along the epilimnion. For this purpose, samples were taken from five depths (subsurface, 0.5, 1, 4

and 7m) and treated as described above for the extraction and HPLC quantification of sestonic microcystins.

6.1.4. Results

Considering temperature profiles, as well as chl *a* and dissolved O_2 profiles (data not shown), stratification was observed in Valmayor and Santillana reservoir. In the case of Cogotas reservoir, massive withdrawal of water resulted in an unstratified watercolumn. In which the water temperature remained above 21 ºC during August, dropping below 18 ºC at the end of September and below 15 ºC in October. Stratification observed at the sampling point in Valmayor reservoir lasted until the end of September. In Santillana reservoir, mixing of the water column at the sampling point began to take place during the first half of October. Prior to mixing, average temperature in the epilimnion of both reservoirs was above 20 ºC. Afterwards temperature dropped rapidly, average water temperature at the end of October was below 16 ºC in both systems.

All three reservoirs showed some episodes of high subsurface chl *a* concentration (fig. 6.1.1). Santillana reservoir hosted massive phytoplanktonic presence from mid August to mid November, when sampling was interrupted. Chl *a* concentrations above 20 μg l^{-1} were frequent during this time, relative abundance of cyanobacteria in these episodes ranged from 27.8% to 87.6%, being more important at the later part of the sampling period. In Cogotas reservoir, cyanobacteria were dominant along the whole sampling period. Maximum chl *a* concentration (116.3 μg l^{-1}) was recorded on August 23rd. In Valmayor reservoir, cyanobacteria were detected, with chl *a* concentrations attributed to this group of up to 9.16 μg l^{-1}. In all cases, the genus *Microcystis* clearly dominated the cyanobacterial community.



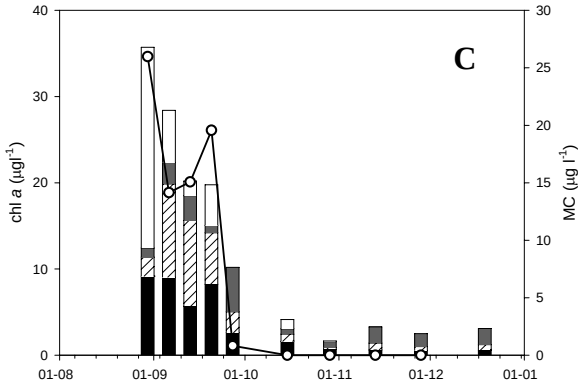


Figure 6.1.1: Algal groups (stacked bars) and sestonic pelagial microcystin concentration (solid line) in subsurface samples in A) Santillana reservoir (2007), B) Cogotas reservoir (2006) and C) Valmayor reservoir (2007). Cyanobacteria (black bar), green algae (hatched bar), diatoms (grey bar) and cryptophytes (white bar) are represented. Scale is not the same in the three graphs

Both the cyanobacterial blooms observed in Valmayor and Cogotas reservoir produced microcystins in subsurface samples (fig. 6.1.1). *Microcystis aeruginosa* and *Microcystis flos-aquae* were respectively present when MC were detected at these reservoirs. These two species were later identified as MC producers by isolation and culture of strains and by MALDI-TOF analysis of single colonies from fresh samples (data not shown). In both cases studied, maximum MC concentrations (25.9 and 64.8 µg l^{-1} respectively) were observed in subsurface samples at the first sampling date. In Cogotas reservoir, concentration then rapidly declined to 1.41 µg l^{-1} in two weeks. In Valmayor reservoir, significant presence lasted up to one month, with a secondary MC concentration peak on September 20th. After this second peak, concentration dropped dramatically in one week as *Microcystis aeruginosa* disappeared from the system. Concerning the different chemical species of microcystins, in Cogotas reservoir the toxic bloom was characterised by the detection of only one variant of microcystin, namely MC-LR. In Valmayor reservoir up to eight different chemical species were observed.

Microcystins in the extracellular fraction were not detected at any sampling date in Valmayor reservoir. In Cogotas reservoir, a concentration of 0.77 µg l^{-1} extracellular MC concentration was detected at the first sampling (August 4th) at subsurface samples. At the same date, one meter above the sediment, extracellular MC concentration was 0.21 µg l^{-1}. Concentration afterwards decreased: Dissolved toxin concentration in subsurface samples and just above the sediment was 0.19 µg l^{-1} and 0.14 µg l^{-1} at August 14th and 0.03 µg l^{-1} and 0.06 µg l^{-1} on August 23rd. In Santillana reservoir, no presence of MC was detected neither in intracellular nor in extracellular samples.

In order to evaluate the possible importance of daily vertical migration in sedimentation values, intracellular MC profiles along the epilimnion were performed at morning, noon and evening during September 25th at Valmayor reservoir (fig. 6.1.2). Vertical migration is indeed observed, bands of maximum MC concentration seem to be homogeneously distributed at morning, then ascend concentrating on surface at noon and again descend at evening.

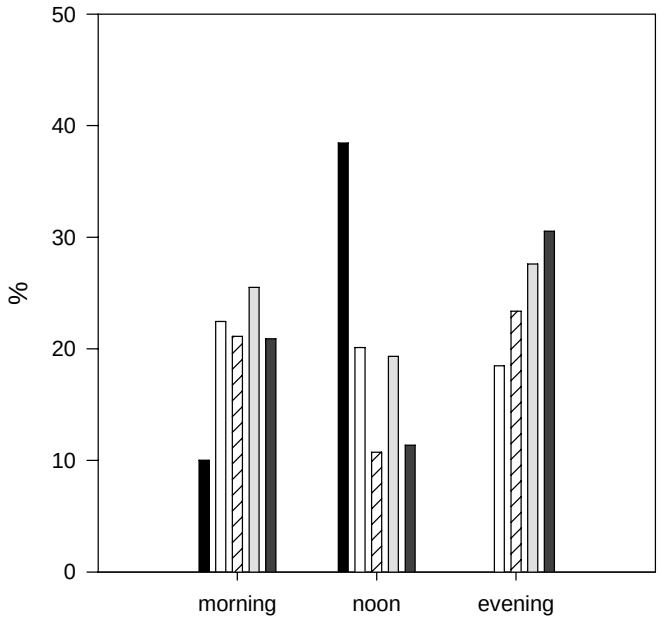


Figure 6.1.2 Distribution of MC along the epilimnion of Valmayor reservoir at morning, noon and evening. Percentages are calculated in relation to the sum of concurrent MC concentrations at the five depths: surface (black bar), 0.5m (white bar), 1m (hatched bar), 4m (light grey bar) and 7m (darkgrey bar)

Sedimentation of both organic and inorganic matter was remarkable along the whole sampling period in all of the reservoirs (table 6.1.2) several grams of organic and inorganic matter settling each day per square meter. Lowest sedimentation rates were observed in Santillana reservoir, while highest values were achieved in Cogotas reservoir, especially concerning OM. It may be of interest that the values observed at each of the reservoirs remain quite constant for most of the sampling period. Also, concerning epi- and hipolimnetic traps, sedimentation rates were similar at both depths. Only at the later part of the experimental period these trends seem to change. In the case of Santillana reservoir, OM and IM recorded at the hipolimnetic trap clearly increased on October 22nd, while in the case of Valmayor reservoir, this increase may be observed

already from September 27th on. In both cases, the IM increase in hypolimnetic traps is especially significant.

		Date	OM (g m^{-2} d^{-1})	IM (g m^{-2} d^{-1})	MC (mg m^{-2} d^{-1})	MC:MO (mg/g)
Santillana reservoir	epilimnetic	01/08/2007	1.49 ± 0.40	0.75 ± 0.16	0.01 ± 0.004	0.0064
		13/08/2007	1.51 ± 0.25	2.63 ± 0.54	0.01 ± 0.007	0.0074
		29/08/2007	---	---	---	---
		11/09/2007	1.32 ± 0.19	1.02 ± 0.11	n.d	n.d
		05/10/2007	0.98 ± 0.10	1.45 ± 0.21	n.d	n.d
		22/10/2007	0.92 ± 0.12	1.55 ± 0.16	n.d	n.d
	hipolimnetic	01/08/2007	1.83 ± 0.64	0.61 ± 0.07	0.01 ± 0.001	0.0049
		13/08/2007	1.57 ± 0.11	2.38 ± 0.31	0.02 ± 0.007	0.0094
		29/08/2007	---	---	---	---
		11/09/2007	---	---	---	---
		05/10/2007	1.19 ± 0.17	0.92 ± 0.07	n.d	n.d
		22/10/2007	3.33 ± 0.32	6.14 ± 0.31	n.d	n.d
Cogotas		14/08/2006	7.27 ± 0.22	9.67 ± 0.13	2.47 ± 0.19	0.34
		23/08/2006	8.95 ± 0.09	13.1 ± 1.43	1.94 ± 0.05	0.22
		07/09/2006	9.85 ± 1.10	20.1 ± 6.09	0.39 ± 0.01	0.04
		03/10/2006	6.73 ± 0.78	13.6 ± 0.15	0.78 ± 0.32	0.11
Valmayor	epilimnetic	06/09/2007	2.71 ± 0.49	1.11 ± 0.33	1.16 ± 0.08	0.43
		13/09/2007	2.81 ± 0.01	0.71 ± 0.06	0.81 ± 0.02	0.29
		27/09/2007	2.47 ± 0.43	3.51 ± 0.19	1.22 ± 0.33	0.49
		15/10/2007	3.30 ± 0.35	4.31 ± 0.34	0.010 ± 0.003	0.003
		29/10/2007	2.03 ± 0.10	2.48 ± 0.35	0.015± 0.001	0.008
		28/11/2007	4.74 ± 0.92	12.4 ± 43.30	n.d	n.d
	hipolimnetic	06/09/2007	3.54 ± 0.41	1.31 ± 0.32	0.43 ± 0.03	0.12
		13/09/2007	2.54 ± 0.22	0.81 ± 0.55	0.83 ± 0.28	0.33
		27/09/2007	7.12 ± 0.02	14.4 ± 2.83	2.53 ± 0.02	0.35
		15/10/2007	5.04 ± 0.44	17.2 ± 3.10	0.55 ± 0.24	0.11
		29/10/2007	3.24 ± 0.29	12.3 ± 3.04	0.28 ± 0.16	0.085
		28/11/2007	3.58 ± 0.47	16.0 ± 2.62	n.d	n.d

Table 6.1.2: Sedimentation rates of organic (OM) and inorganic matter (IM) and microcystins (MC) and ratio of settling microcystins:organic matter in Santillana, Cogotas and Valmayor reservoir. (n.d.: not detected)

Concerning MC in the traps (table 6.1.2), in Santillana reservoir, where MC had not been detected in the water column, it was observed in small amounts in both epi- and hipolimnetic traps during the first half of August. Calculated MC sedimentation rates in this reservoir range from 0.012 to 0.020 mg m^{-2} d^{-1}, while the MC:OM ratio in the traps ranges from 0.0049 to 0.0094 mg g^{-1}. In Cogotas and Valmayor, toxin sedimentation was high, up to 2.47 and 2.53 mg MC m^{-2} d^{-1} were respectively measured. During the toxic bloom episodes, the ratio MC:OM in the traps ranged from

0.12 to 0.43 mg g^{-1}. Afterwards this ratio strongly decreased to values more similar to those observed at Santillana reservoir.

Interestingly, the high diversity of chemical species of MC in sestonic samples from Valmayor reservoir is also found in samples from the sediment traps. Figure 6.1.3 shows representative chromatograms from subsurface samples at the first sampling date (A) and from epilimnetic traps on September 27th, when the toxic bloom had already declined (B).

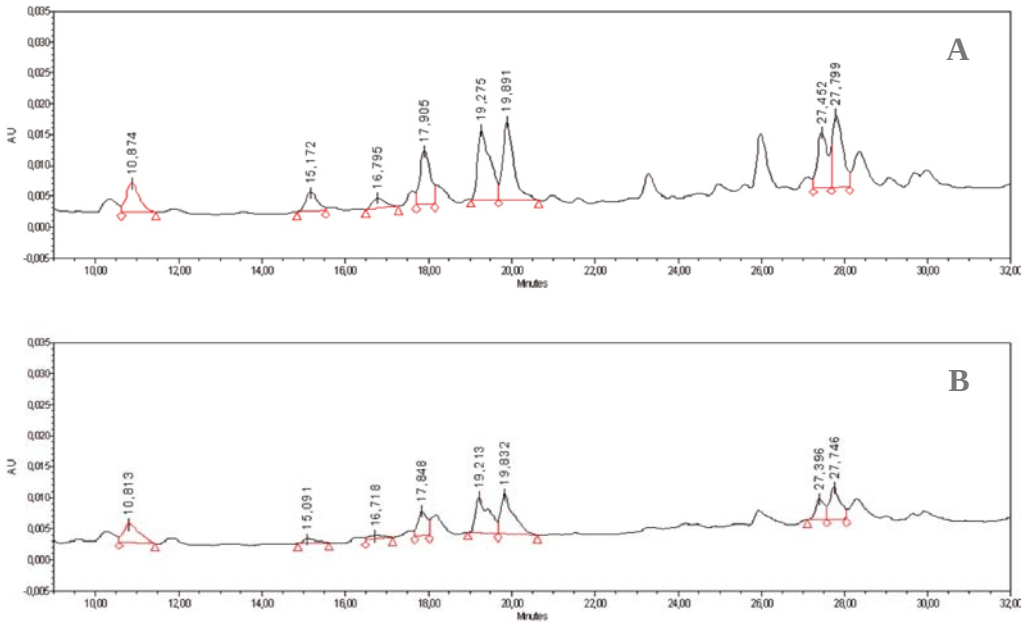


Figure 6.1.3: HPLC chromatograms of microcystins in samples from Valmayor reservoir A) sestonic epilimnetic sample on August 30th and B) hipolimnetic trap on September 27th

Having a closer look at intracellular microcystins along the watercolumn and in the traps (fig. 6.1.4), we find that in Cogotas reservoir most of the toxin is always found in the subsurface samples. During the second sampling date (Aug 14th) though, a considerable presence of sestonic MC (3.46 µg l^{-1}) is observed at the depth where traps had been deployed, this concentration is equivalent to 8% of the concurrent toxin concentration in subsurface samples. Simultaneously, highest sedimentation rates were observed at this sampling date. One week later, when the final drop of sestonic concentration in the system was taking place, sedimentation slightly decreased.

A similar scenario may be observed at Valmayor reservoir (fig. 6.1.4 B). During the toxic bloom, August 31st to September 27th, most of the toxin is found in subsurface samples. Nevertheless, sestonic MC in the watercolumn surrounding the epilimnetic traps is relevant during some episodes. While this concentration accounts for 7% of the concurrent subsurface concentration during the first pelagial sampling, one week later its importance is increased to 32.7%. Importance declines to 8.5% after another week (Sept 13th) and to only 0.8% another two weeks later (Sept 27th). In the water surrounding the hipolimnetic traps, MC concentration is generally very low. Only on September 6th and 13th, concentrations slightly increased, reaching 0.41 and 0.53 μg l^{-1}, which accounts for 2.91 and 3.54% of toxin in concurrent subsurface samples. Considering sedimentation rates, these are constantly high during the bloom period. When comparing epi- and hipolimnetic traps, more toxin is initially recorded at the upper traps. Sedimentation rates at the second trap sampling date are almost identical and finally at the third sampling (September 27th), hipolimnetic trap MC content is clearly increased. This dynamic seems closely related with the varying presence of MC in the trap-surrounding water, providing an approximate idea of the travel of toxic cells along the watercolumn. Still, as will be discussed later, the heavy increase of toxin in the hipolimnetic traps on September 27th may also be related to resuspension processes.

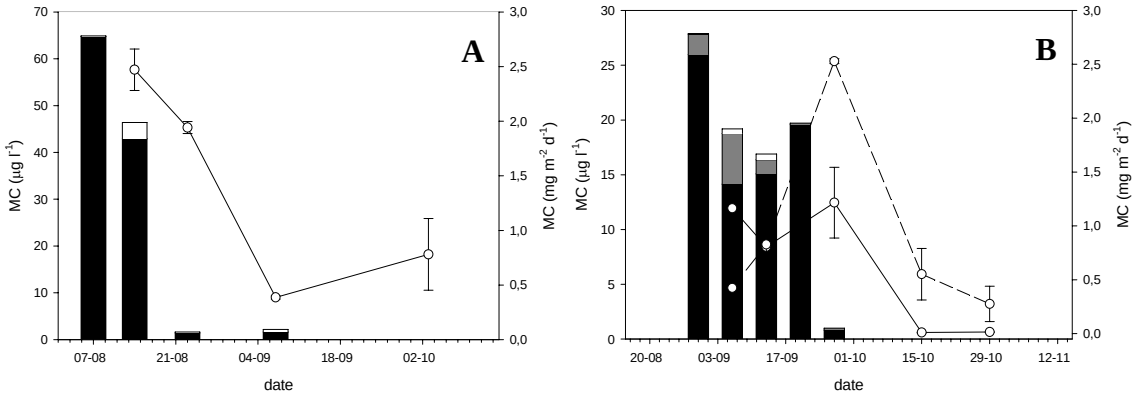


Figure 6.1.4: Microcystins in Cogotas (A) and Valmayor (B) reservoirs. Total sestonic pelagial microcystin concentration along the watercolumn is shown by stacked bars: subsurface (black bar), deep epilimnion (gray bar) and one meter above the sediment (white bar). Lines show sedimentation rate of microcystins, in Valmayor: epilimnetic trap (solid line) and hipolimnetic trap (dashed line).

The higher resolution sampling in Valmayor reservoir at August 30th (data not shown), as well as the study of daily migration along the column, allowed us to have a more complete understanding of the expected vertical distribution of sestonic pelagial MC in the reservoir. With the help of these data and the remaining sampling data, a reconstruction of toxin distribution along the whole watercolumn during the sampling period in Valmayor reservoir was constructed (fig. 6.1.5). This reconstruction allows us to estimate the concentration of microcystin expected at any given depth and moment.

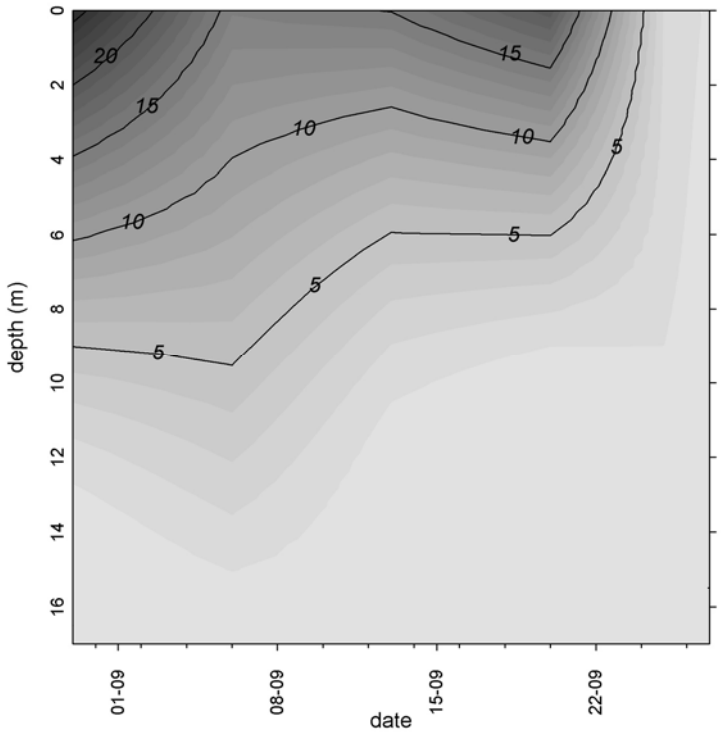


Figure 6.1.5: Estimated distribution of sestonic pelagial microcystins (µg l^{-1}) during the sampling period along the 16 m water column in Valmayor reservoir.

This estimation is important in order to evaluate the importance of the described sedimentation processes, as we are able to calculate the amount of toxin in the watercolumn above the sedimentation area of a given trap and along a given time period. The comparation of these values to the amount of MC recovered by the trap allows us to estimate the fraction of toxin that has settled in that period. Our estimations (table 6.1.3) show that an average of 4.45% of the toxin in the epilimnion is settling at any given moment during the *Microcystis* dominated bloom in Valmayor reservoir. If considering the whole watercolumn, including meta- and hipolimnion, this percentage increases to an average 4.75%.

	01/09 – 06/09/2007	06/09 – 13/09/2007	13/09 – 27/09/2007
Epilimnetic traps	0.047	0.046	0.040
Hipolimnetic traps	0.016	0.044	0.082

Table 6.1.3: Importance of microcystins settled in epi- and hipolimnetic traps deployed at Valmayor reservoir. Estimated ratio of settled MC:overlying sestonic MC is shown for each sampling interval.

6.1.5. Discussion

The results obtained in all three reservoirs studied clearly show the great importance of sedimentation processes in the fate of microcystins. During MC-producing blooms, the amount of toxin settled per day and square meter can be expected to be in the range of milligrams. Obviously, this value will strongly depend on the toxicity and dominance of the MC-producing organisms. It may be interesting to describe that even when MC is not detected in the watercolumn due to very small concentrations, as was the case of Santillana reservoir, the toxin may be found in epi- and hipolimnetic sedimentation traps. Sedimentation may be acting as a concentration process of microcystins, being able to turn undetected toxins into visible risk. Also, it seems interesting to compare the magnitude of MC sedimentation rates and extracellular MC concentrations. In Valmayor reservoir, with both high intracellular MC concentration and high MC sedimentation occurring, extracellular toxin could not be detected, possibly due to limited cell lysis and efficient dilution and degradation. In Cogotas reservoir, which due to major withdrawal had limited depth, extracellular concentration achieved maximum values of 0.77 μg l^{-1}, accounting for only 1.2% of concurrent intracellular concentration.

In Valmayor reservoir, due to higher sampling resolution, we were able to estimate the importance of the sedimentation process on the overall MC budget. These estimations showed that about 4.5% of MC in the watercolumn may be involved in sedimentation at a given timepoint. Such values clearly show the importance of these processes and are even slightly above those estimated by Verspagen et al. (2005) by mathematical modelling for *Microcystis* colonies, which – even in the scenario of highest sedimentation – remained below 3%.

Beside this, the high ratio of MC to overall organic matter in the traps is worth mentioning. For example, in Valmayor reservoir, where MC producing organisms are not dominant, MC:OM ratios ranged from 120 to 490 µg/g. In bloom or water samples, highest MC:Dry Weight ratios described in the literature are around 7000 µg/g (Vasconcelos et al., 1996; Zhang et al., 1991), while usually values are much lower (Sivonen and Jones, 1999). Here the given ratio considers MC in terms of overall settling organic matter. This clearly shows that a high amount of highly toxic *Microcystis* cells are settling and suggests that massive loss of intracellular MC is not occurring. Still, it is surprising that the MC:OM ratio seems to decrease in the hypolimnetic traps in comparison to epilimnetic traps. Possible explanations for such behaviour could be a slight liberation of MC due to lysis of some producing cells while settling or the collection of MC-free biomass at the hypolimnion. Also, this phenomenon may be explained by the regulation of MC production by the cells. Toxin production has been shown to be limited by cell division rates (Jones and Orr, 1998), while at the same time high light intensities seem to trigger the transcription of the MC biosynthesis gene cluster (Kaebernick et al., 2001). Both factors could be negatively affecting MC production in settling colonies.

Observing the obtained sedimentation patterns in more detail, it is remarkable that sedimentation processes are not mainly occurring during the decay of the bloom, but almost constantly during the whole bloom period. Usually, *Microcystis* sedimentation is related with decreasing water temperature, thus autumnal sedimentation is expected to be most important (Visser et al., 1995). Our data show that MC sedimentation is occurring constantly at high rates and even with surface water temperature above 20 ºC. Similar results have been described by Ihle et al. (2005), who observed that during a period of almost three months presence of benthic *Microcystis* and MC strongly increased, the late phase of pelagic growth overlapping with the early phase of sedimentation. Further, this major presence of *Microcystis* and its toxins in the sediment was observed to take place from August onward, an observation that is perfectly consequent with the sedimentation processes described here. Sedimentation of MC might thus be considered a biphasic process, including constant sedimentation during the bloom period and, if the toxic community persists, enhanced autumnal sedimentation.

Microcystin concentrations along the watercolumn and in the traps seem to tell us a tale of constant vertical travelling. This travel is best observed in the data concerning Valmayor reservoir: initially, while the bloom is developing, toxin is found mainly in subsurface sestonic samples, a week later, sedimentation has begun to take place massively, important epilimnetic sedimentation along with increased sestonic MC in surrounding water is observed, while hipolimnetic sedimentation is still weak. Another week later, epilimnetic sedimentation keeps on, while previous sedimentation seems to be arriving in the hipolimnion, epi- and hipolimnetic sedimentation equilibrating. This would indicate a time lag of around two or three weeks between initial settling of pelagial MC and its arrival at sediment surface. Such time lags are similar to those estimated by Verspagen et al. (2005) for *Microcystis* populations for shallow sites of lake Volkerak, but significantly lower than those estimated for the sites of similar depths to those of the waterbodies studied here. As water temperature in our studies is close to the temperature observed by Verspagen et al. (2005), this more rapid vertical travel of the *Microcystis* colonies and the toxin within has to be explained by means other than higher water density.

In Valmayor reservoir, MC producing organisms were responsible for the sestonic production of up to eight chemical species of MC. It is interesting to note that the MC profile in watercolumn and sediment trap samples is almost identical and stable over the whole toxic bloom period (fig. 6.1.3). This, on the one hand, confirms the pelagial origin of settled MC during the whole sampling period and is in accordance with the studies of Welker et al. (2007) when studying seasonal shifts in chemotype composition of *Microcystis*. They observed that chemotype composition in sediment samples from November was very closely related to pelagial chemotypes from the previous summer bloom. On the other hand, no selective sedimentation of the different MC variants seems to be occurring. The diverse chemical species of MC pose very different properties in aspects as could be hydrophobicity. A selective sedimentation thus could indicate liberation or adsorption processes. As none of this seems to be occurring, we could hypothesize that the observed sedimentation of MC is mainly due to sedimentation of intact cells. Also this would be in accordance with the results from Welker et al. (2007) when they interpreted that similar detectability of peptides in benthic and pelagial samples points towards integrity of *Microcystis* cells during the

benthic stage. This hypothesis is also sustained by the extremely low extracellular MC concentrations observed.

We considered it of main interest to asses the influence of daily movement processes of the cyanobacterial community on the measured sedimentation rates. These communities are expected to keep ascending during the early hours of the day (van Rijn and Shilo, 1985), a behaviour that also was observed in our case when studying daily distribution of MC due to vertical migration of producing organisms. By sampling between 11 and 12 a.m. we avoided cyanobacteria subjected to daily movement to be retained in the deep epilimnetic traps. Further, the closely linked data from epi- and hipolimnetic traps tell us that the sedimentation at epilimnetic traps have a continuation in the hipolimnion, the observed sedimentation may thus be considered to be irreversible.

Concerning other factors that may be affecting the robustness of our data, resuspension may be influencing the sedimentation rates estimated for hipolimnetic traps at some concrete dates. In both Santillana and Valmayor reservoir, the final sampling dates show a strongly enhanced recovery of organic and, especially, inorganic matter in the hipolimnetic traps, an increase that is not accompanied by a similar increase in the epilimnetic traps. As rupture of stratification is observed close to these dates, the source of such enhanced hipolimnetic trap content may be, in some extent, related to concrete resuspension processes. Therefore the MC sedimentation rate of 2.53 $\mu g\ l^{-1}$ and the estimation of 8.2% of toxin being involved in settling processes at Valmayor reservoir at September 27th should be treated with care.

Our results suggest a constant toxic rain of more or less intact MC containing organisms during bloom episodes. This will result in an important accumulation of MC at the sediment surface, most of the toxin still being caged inside the cells. It will be crucial to understand further processes these toxins will be subjected to in order to correctly evaluate associated risks. Benthic *Microcystis* has been described as being able to maintain cellular integrity during long periods (e.g.: Latour et al., 2007). These intact cells will be essential for recruitment during the next spring (e.g.: Verspagen et al., 2005), but also may be considered an important direct risk in reservoirs in which water is usually withdrawn in depth. Resuspension of these MC containing organisms may open a direct way for the toxin towards human consumption.

Even if *Microcystis* is capable of surviving within or upon sediment during long periods, some colonies or cells may be suffering grazing (Boström et al., 1989) or lysis. In this case, the intracellular toxin could be liberated. For example, Song et al. (2007) recently observed extraordinarily high extracellular MC concentrations (6.69 μg l^{-1}) in very shallow lake Taihu. Such massive presence in the dissolved state may be explained by lacking sequestration of the toxin in sediment and by liberation of MC from both pelagic and benthic organisms. This could be supported also by the fact that Chen et al. (2008) described a larger number of MC degrading bacteria in the sediments of shallow lake Taihu than in the water, a fact that points towards important presence of available MC in the sediments. In such systems, the sediment could be an important source of high temporary extracellular microcystin concentration, as the toxin may be liberated to the water without the possibility of major dilution. Also, such behaviour explains why extracellular MC in our studies is only found in Cogotas reservoir. This waterbody was of very limited depth, as massive water withdrawal had been carried out, therefore dilution was not as efficient. Also, the fact that extracellular concentration is similar in subsurface samples and in samples from just above the sediment suggests that both pelagic and benthic colonies may be liberating toxins. In such shallow systems, sedimentation might act more as an additional source for microcystins than as an actual sink. In deeper systems, where sediments may become anoxic, biodegradation in the sediments will be limited (Holst et al., 2003) and a decrease of extracellular MC concentration will rely almost entirely on dilution. If withdrawal is close to the sediments, MC producing organisms in these sediments may not only pose a risk due to resuspension of MC containing cells, but also due to liberation and limited degradation of intracellular toxin.

6.1.6. Acknowledgement

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6.2. Presence and detection of cylindrospermopsin and microcystins in reservoir sediments

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6.2.1. Abstract

Cyanobacterial toxins have been a concern for decades due to worldwide appearance of toxic blooms. Still, their presence in sediments of lakes or reservoirs have received less attention. In our work, we focused on two kinds of cyanotoxins, microcystins and cylindrospermopsin, and studied their adsorption behaviour on two natural sediments and commercial clay and their further extraction for quantification. Cylindrospermopsin sorption was very low under the conditions tested, and seems unaffected by the presence of clay, thus suggesting limited importance of this phenomenon in the field. In the case of microcystins, potential sorption to clay is very important, but we keep some reservations concerning the possibility of such adsorption occurring in the field. Our data show that adsorption to fresh sediments is extremely low and only taking place in the case of MC-RR. Only dehydration or mineralization of sediments seems to activate adsorption processes. Therefore we suggest that, in the field, MC inside producing organisms may be of greater concern than MC adsorbed to inorganic particles. Further, available extraction techniques were tested, results not being satisfactory neither in the recovery of spiked MC nor in the detection of naturally occurring MC, possibly due to both strong adsorption of MC to clay and presence of a great number of other UV-absorbing compounds. Considering that best results were obtained by separation of MC producing cyanobacteria by density gradient centrifugation, we suggest that future MC extraction methods should include processes that allow to either separate MC containing particles or clean up steps that allow a better detection of MC in the crude extracts.

6.2.2. Introduction

Cyanobacteria are known to show a very active secondary metabolism. Many of these metabolites have been described as bioactive compounds, and some among them are of concern because of their toxicity to diverse organisms (Carmichael, 1992). Among these cyanobacterial toxins, the microcystins (MC), a family of cyclic heptapeptides, are best studied. The number of chemical species included in this family, originated mainly by the variable occupation of two aminoacid sites, is constantly increasing, and, for example, Codd et al. (2005) recorded 71 variants. Microcystins are produced and retained in the cells of producing organisms, and apparently only released in greater amount during senescence (e.g.: Wiedner et al., 2003; Rohrlack and Hyenstrand, 2007). Further, it has been described that in the most notable MC-producers, namely the genus *Microcystis*, sedimentation of colonies is important along most of the bloom period, the late phase of pelagic growth overlapping with the early phase of sedimentation (Ihle et al., 2005). Also, our previous studies (Wörmer et al., submitted) have shown that these sedimentation processes may be acting as a very important vertical flux of microcystins inside cells of producing organisms. Therefore, high amounts of the toxin may be expected in the sediments, and a complete knowledge of their fate in these sediments may be considered of greatest interest.

Cylindrospermopsin (CYN) is a small, toxic alkaloid which has been found in high extracellular concentration in environmental samples (Wörmer 2009 and references within). Such high presence has been explained by matters of enhanced liberation (Mihali et al., 2008; Preussel et al., 2008) but also due to limited *in situ* degradation (Smith et al., 2008; Wörmer et al., 2008). Cylindrospermopsin is produced by diverse filamentous cyanobacteria, as for example *Cylindrospermopsis raciborskii* (Ohtani et al., 1992), *Aphanizomenon ovalisporum* (Banker et al., 1997) or *Aphanizomenon flos-aquae (*Preussel et al. 2006). As massive liberation of the toxin is expected even during vegetative growth, CYN, in principle, may rather be expected in the sediments due to adsorption to settling organic or inorganic matter than inside akinetes and complete filaments.

Even though sedimentation has long time been identified as a crucial step in the annual cycle of some toxic cyanobacteria (Reynolds, 1980, Preston et al., 1980), less attention has been paid to cyanotoxins in the sediments when compared to sestonic toxic

blooms. In the case of MC, some authors have explored the adsorption potential of soils and sediments and concluded that such sorption depends directly on clay content, and that naturally occurring clay particles have a great potential of MC sequestration (Morris et al., 2000; Miller et al., 2001). This capacity of soils and sediments to scavenge MC from solution has been interpreted as a clear sign that toxin could be accumulating adsorbed to these solids in sediments of lakes and reservoirs. Therefore in 2001, Tsuji et al. made first attempts to establish an effective extraction method for microcystins from sediment samples. Afterwards, Babica et al (2006) or Chen et al. (2006a) have proposed diverse HPLC-methods which should be able to recover these toxins from spiked sediments. In the case of CYN, to our knowledge, no publications regarding their presence in or extraction from sediments are available.

In this work, we tried to obtain a better understanding of MC and CYN dynamics in sediments, by A) studying their sorption behaviour B) testing diverse extraction methods and C) applying such methods to environmental samples in which the studied toxins could be expected to be found in recent sediments.

6.2.3. Materials and Methods

Pure cylindrospermopsin and microcystins were obtained from Alexis Biochemicals (San Diego, USA). Cylindrospermopsin and microcystin extracts were obtained from exponentially growing cultures of *Aphanizomenon ovalisporum* UAM 278 (CYN) and *Microcystis aeruginosa* UAM 247 (MC-RR, MC-LR and MC-YR). HPLC analyses of field and experimental samples were performed on a HPLC-PDA system (Alliance, Waters) consisting of a Waters Separations Module 2695, equipped with a Waters 996 PDA following the procedure proposed by Lawton et al. (1994). The chromatography column was a Purospher RP 18 endcapped (5µm, 4.6 mm x 250 mm) column. Chromatograms were monitored at 238 nm and toxin concentration determined by comparison to the injected standards for MC-LR, RR and YR. For CYN, the system was equipped with a Waters Spherisorb 5µm ODS2 column (4.6 mm x 250 mm) and analysis were performed according to the protocol described by Törökné et al. (2004). The presence of CYN in the field samples was verified by its UV spectrum and its retention time and quantified by comparison to injected standards

6.2.3.1. Adsorption of MC and CYN

Adsorption experiments consisted of the exposure of 10 ml sterilized, diluted toxin solution (0.9% NaCl) to 0.5 g of the selected sorbent. Three different sorbents were used: sediments from Santillana reservoir and Salas reservoir and clay (benthonite, Acros Organics). Sediments were used in adsorption experiments as obtained, freeze-dried and mineralized (4h, 500 ºC). Further, sediments were used rewetted or non-rewetted. Rehydration consisted in incubating the sorbent in 9ml sterile saline solution (0.9% NaCl) during 48h under continuous shaking, before addition of the toxin. Afterwards 1 ml filter sterilized spiking solution was added and incubated for 24 h. In the case of non-rewetted sediments, toxin was spiked together with the saline solution.

Santillana reservoir is located close to the city of Madrid (Spain). Sediments were collected on October 5th 2005 with a Kajak sediment corer (Uwitech, Austria) and cut in 2 cm layers. Layers from the upper 7.5 cm were homogenized and kept at 4 ºC. Salas reservoir is located in North Western Spain, in the Orense region. In this case, sediments were collected on February 19th 2007 and the upper 5 cm of the core were used.

A first adsorption experiment was conducted for 7 days and included CYN, MC-RR and MC-LR. This experiment was performed on rewetted, freeze-dried and mineralized sediments and on benthonite (Acros Organics), all having been rewetted before addition of the toxin. Final concentrations were 10 μgl^{-1} for pure MC and CYN added to clay; and 11.43 μgl^{-1} and 14.7 μgl^{-1} for CYN and MC extracts added to the sediments. Toxin remaining in the dissolved state was quantified at selected incubation times after filtration (0.45 μm) by HPLC-PDA as described below.

A second 24h-experiment studied the adsorption of MC-RR, -YR and –LR in more detail. Therefore, sediments from Santillana reservoir were used fresh, freeze-dried and mineralized. The dry and mineralized samples were used both rewetted and non-rewetted. Initial MC concentration in these studies was three times higher in order to be able to detect MC-YR. Negative controls were obtained for each experiment by not adding any solids to the spiked saline solution.

6.2.3.2. Recovery of spiked MC

Once adsorption experiments were completed, an attempt to recover MC from sediments from Santillana reservoir was performed. Two different MC-extraction procedures were tested on these samples: firstly, following the method proposed by Chen et al. (2006 a), an EDTA pyrophosphate solution (0.1 M EDTA 0.1 M $Na_4P_2O_7$) was applied. Secondly, a more traditional approach, based on acidified (0.1% TFA) aqueous methanol (90%), was tested. In both cases, ultrasonication (Branson Sonifier 450) was applied at highest power for microtips in 70% duty cycles.

6.2.3.3. Extraction of MC and CYN from natural samples

Finally, extraction of toxins from natural sediments was studied. Cylindrospermopsin was expected in the sediments of Arcos reservoir, where high concentrations had been detected in sestonic samples during the summer 2004 (Quesada et al., 2006) and in lower concentration in the year 2005. In the summer 2008, we sampled Arcos reservoir and took sediment cores at three sampling points. Sediment cores were also collected at Bornos reservoir. This reservoir provides water to Arcos reservoir and has been suggested to be the source of CYN-producing *Aphanizomenon ovalisporum* in Arcos reservoir (Quesada et al., 2006). Three cores were taken in each reservoir and cut into ten 2 cm layers. Two aliquots were taken from each layer and each extracted three times by ultrasonication, one being extracted into aqueous methanol (90%) and the other one into saline solution (0.9% NaCl) in the presence of 5% formic acid as described by Torokne et al. (2004) for sestonic samples. These extractions were afterwards dried under vacuum, resuspended in Milli-Q water and prepared for HPLC-PDA analysis.

Microcystins were extracted from nine reservoirs in North-West Spain. Sestonic subsurface water samples were taken in all reservoirs in the summer 2007, and in the case of Trasona reservoir in both 2006 and 2007. Additionally, sediment samples from all reservoirs were taken in January and February of the years 2007 and 2008. In each reservoir, two sampling points were chosen: close to the dam and upstream. Sestonic samples were GF/F filtered, filters were stored frozen (-20 ºC) and extracted into aqueos methanol (90%). For sediment samples, the upper 5 cm layer of each sample were homogenized and kept frozen. Extraction procedures tested were those described above

(EDTA pyrophosphate and acidified methanol). In addition, in samples from the year 2007, extraction in 5% acetic acid and 0.2% TFA (trifluoroacetic acid) as described by Babica et al. (2006) was performed. In the 2008 samples, this extraction was substituted by a method including density-gradient centrifugation: 3 g of fresh sample was added to 25 ml of centrifugation medium (90% Percoll, 10% Sacarose 2.5 M) and centrifuged for 15 min at 4 ºC and 20.000g. This centrifugation allowed to separate cyanobacteria, which remained in the upper layer of the supernatant, from the sediment. These cyanobacteria were afterwards retained on GF/F filters and extracted as sestonic samples (90% methanol).

6.2.4. Results

6.2.4.1. Adsorption of MC and CYN

Adsorption experiments with the two selected sediments (fig. 6.2.1) first of all show that adsorption, when taking place, occurs rapidly. After 24 h, non-adsorbed compounds remain in dissolution.

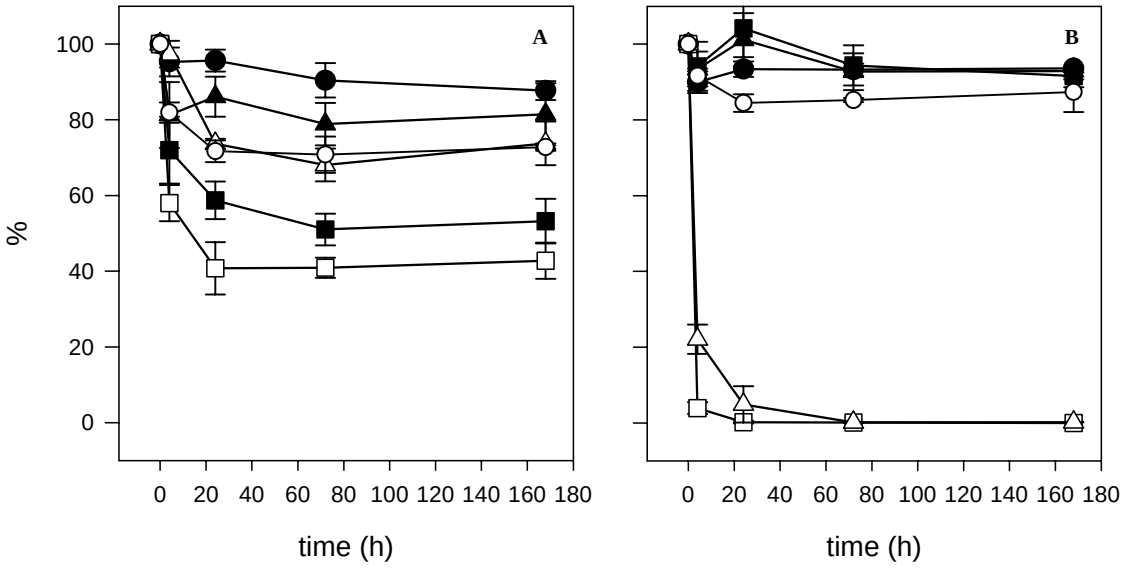

Figure 6.2.1 Over-time adsorption of CYN (circles), MC-RR (square) and MC-LR (triangle) from Santillana (white fill) and Salas (black fill) reservoir to freeze dried (A) and mineralized (B) sediments. Y-Axis shows percentage of toxin remaining in solution. Mean and standar desviation are shown (n=3)

The experiments conducted with dried natural sediments and crude toxin extract allowed establishing very clear differences concerning the sorption affinity of the tested toxins: MC-RR>MC-LR>CYN (fig. 6.2.1 A). Further, it seems as if the sediments from Santillana reservoir allow more adsorption. In the dry sediments of this reservoir, about 60 and 30% of spiked MC-RR and MC-LR is scavenged from solution.

In the case of mineralized sediments (fig. 6.2.1 B), CYN seems completely unaffected by the presence of this potential sorbent, more than 85% remaining dissolved. On the other side, complete adsorption of both MC species is occurring when exposed to sediments of Santillana reservoir. In the case of Salas reservoir, combustion of these sediments resulted in a complete loss in adsorption capacity, thus suggesting the importance of the composition of the inorganic fraction.

This led towards testing adsorption of MC and CYN on one of the most active components of the inorganic fraction: clay, namely benthonite (fig. 6.2.2). Again, a very rapid adsorption is observed, dissolved toxin concentration did not decrease after 24 h. Further, the different behaviour of CYN and MC is confirmed. While MC completely disappeared from the free fraction already after 4 hours, dissolved CYN seems to be unaffected by the presence of benthonite, concentration remaining above 85% of initial concentration.

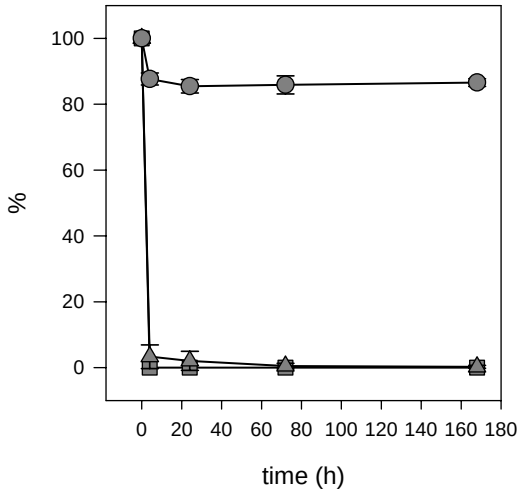

Figure 6.2.2 Over time adsorption of CYN (circles), MC-RR (square) and MC-LR (triangle) on clay. Y-Axis shows percentage of toxin remaining in solution. Mean and standar desviation are shown (n=3)

In addition to these time course experiments, sorption behaviour after 24 h exposure was tested for three chemical species of MC on the sediments of Santillana reservoir, paying special attention to the previous treatment of these sediments. Sediments were used fresh, freeze-dried (rewetted and not rewetted) and mineralized (rewetted and not rewetted) The great variability of the extent of adsorption is easily noticed (fig. 6.2.3). As seen above, MC-RR shows a strong tendency towards adsorption. On fresh samples, 75.5% of initial concentration remains in the dissolved state, this fraction is reduced to 40.7% on freeze dried samples and is no longer detectable on mineralized sediments. Free MC-LR and –YR concentration, on the other hand, are not affected by the presence of fresh sediment. When exposed to dry sediments, the soluble fraction is reduced to 73.7 and 76.4% respectively. Finally, also in this case, mineralized sediments seem to be responsible for a major adsorption of microcystins, as free concentration is reduced to 6.5% and 7.6% respectively. Concerning previous rehydration of the sediments (data not shown), no significant difference in free toxin concentration was detected between rewetted and non rewetted sediments (one way ANOVA, $\alpha = 0.05$).

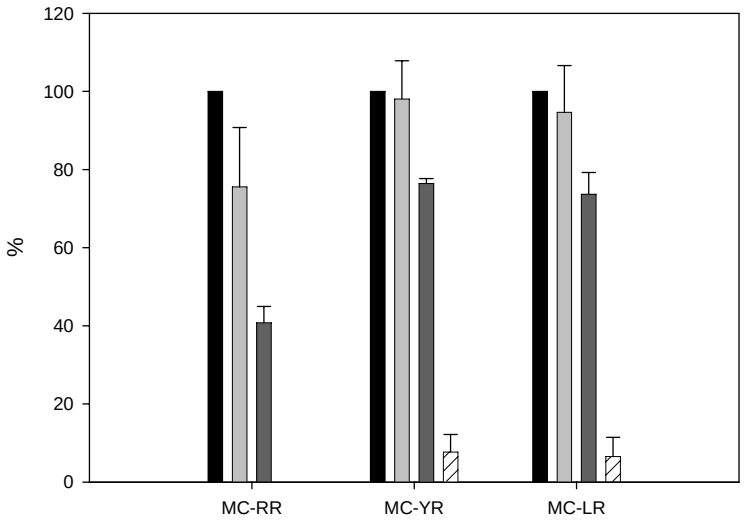

Figure 6.2.3 Initial spiked concentration (black bar) and free concentration after 24 h incubation in presence of fresh sediment (light grey bar), freeze-dried rewetted sediment (dark grey bar) and mineralized rewetted sediment (dashed bar) . Y-Axis shows percentage of MC remaining in solution. Mean and standar desviation are shown (n=3)

6.2.4.2. Recovery of spiked MC

After demonstrating the adsorption capacity of sediments from Santillana reservoir, attempts to recover the toxin were performed (table 6.2.1). Concerning mineralized samples, satisfactory values were achieved for MC-LR by methanolic extraction, while measured recovery of MC-RR was only 18.4%. The EDTA based method yielded recoveries for MC-LR of only 5.4%. Even more surprising is the behaviour of the MC adsorbed to freeze-dried sediments. In these cases no recovery of adsorbed MC could be observed, neither by methanolic extractions nor the EDTA-pyrophosphate based method.

		Methanol	EDTA
Dried	MC-RR	---	---
	MC-LR	---	---
Mineralized	MC-RR	18.2%	---
	MC-LR	74.8%	5.4%

***Table 6.2.1: Measured recovery of MC-RR and –LR from freeze-dried and mineralized sediments from Santillana reservoir expressed as % of adsorbed* toxin after 24 h incubation**

6.2.4.3. Extraction of MC and CYN from natural samples

Concerning toxins naturally present in sampled sediments, the attempt to extract CYN from Arcos and Bornos reservoir did not succeed. Ten layers of each of six sediment cores were extracted in two different ways (ultrasonication into methanol and acidified saline solution) without being able to detect any CYN. In the case of MC, four of the sampled reservoirs were affected by MC-producing summer blooms in one or both of the studied years (table 6.2.2). Highest concentration were obtained in Cachamuiñas reservoir, while Trasona reservoir was the only waterbody sampled in 2006 and 2007, and showed presence of MC in both years. Concerning MC in the sediments, only one sample was found to host MC, namely the upstream sample from Trasona reservoir collected in winter 2007/2008. A MC concentration of 0.41 μg g^{-1} dry weight was detected. It should be noted though, that the toxin was only detected when applying density-gradient centrifugation. The use of the EDTA or methanol based

methods did not allow the detection of MC in this sediment, which were detected by density gradient centrifugation.

6.2.5. Discussion

Very different sorption behaviour was observed between MC and CYN, the latter was poorly adsorbed by tested reservoir sediments and clay. This is important as CYN is expected not to remain inside producing organisms, but rather in the dissolved state (Wörmer et al., 2009 and references within) and thus exposed to settling organic or inorganic particles. According to our results, adsorption of CYN to inorganic particles is neglectable and adsorption to organic particles, although slightly higher, is also occurring at only very limited extent. This is in accordance with the data from Klitzke et al. (2009), who observed that retention of CYN to sandy sediments was neglectable. Therefore, we suggest that CYN might rarely be found in sediments. Indeed, our results from Arcos and Bornos reservoir show that in sites affected by CYN producing blooms, we were unable to detect any toxin. Further research should still be performed analyzing CYN in sediments, and possible focusing on CYN in akinetes and CYN adsorption to organic particles. Results obtained so far might exclude sedimentation as a major sink for CYN, but require further experimental confirmation.

The case of microcystins, and especially MC-RR, is clearly different. These compounds have proven a great affinity for clay, as may be observed in the rapid scavenge of both MC-RR and MC-LR from solution (fig. 6.2.2). Diverse authors had previously suggested that sorption capacity directly depended on clay content (Morris et al., 2000; Miller et al., 2001; Chen et al., 2006b). Also, our observations that MC-RR is more easily adsorbed than MC-LR are consistent with the findings of Chen et al. (2006b). In our study, we were able to characterize the adsorption behaviour of one further chemical variant, MC-YR, which is similar to the one of MC-LR. Chen et al. (2006b) explained the higher adsorption of MC-RR with the presence of additional available binding sites in the form of three more nitrogen atoms. This explanation would also fit our observations concerning low adsorption of MC-YR.

		Seston			Sediment				
		2006	2007		2007			2008	
Extraction method		MeOH	MeOH	EDTA	MeOH	5% acetic acid	EDTA	MeOH	centrifugation
Cachamuiñas	Dam	n.a.	**9.90**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Upstream	n.a.	**1.20**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Castrelo	Dam	n.a.	**0.11**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Upstream	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Frieira	Dam	n.a.	**0.17**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Upstream	n.a.	**0.25**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trasona	Dam	**2.52**	**6.07**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Upstream	**2.65**	**4.55**	n.d.	n.d.	n.d.	n.d.	n.d.	**0.409**

Table 6.2.2: Microcystin in sestonic samples (μg l^{-1}) and sediments (μg g^{-1}) from reservoirs in North Western Spain. Only those reservoirs in which MC was detected in seston and/or sediments are shown. Extraction methods as described in the text.

The MC-adsorption potential of clay seems well established, but we keep some reservations about such adsorption occurring in the environment. Our results show that drying or mineralization of sediments is responsible for activating adsorption processes. In fresh samples, not subjected to any dehydration processes, adsorption is minimal. Many studies are available describing how dehydration may alter the structure of soils and sediments, and thus change their functionality, resulting in positive or negative variation of sorption capacity. Liu and Lee (2006) showed that while sorption of lysine decreased when drying sediments, sorption of tyrosine, aniline or naphtalene clearly increased. Kang et al. (2008) observed that complexation of dicarboxylic acid with goethite changed as dehydration occurred, the new complexation being very stable and not reversible after rehydration. To our knowledge, the studies concerning the evaluation of adsorption potential of MC have been mainly carried out on soils (Miller et al., 2000; Chen et al., 2006b), with lower water content, or on freeze-dried sediments, which were not rewetted before application of the toxin (Babica et al., 2006; Chen et al., 2006a; Mohamed et al., 2007). The results obtained by working with dry soils or dehydrated sediments could be giving some useful information concerning potential adsorption to clay or soils, or the importance of wetting-drying cycles on bioavailability of MC, but may be somehow misleading when estimating the importance of adsorption under natural conditions.

Some other aspects may be considered when estimating naturally occurring MC adsorption. Beside the importance of hydration mentioned above, also the effect of organic carbon (OC) on MC-clay interaction is far from clear. While Miller et al. (2001) related highest adsorption with both highest clay and OC content, Chen et al. (2006b) observed a different behaviour, as high OC content seemed to benefit MC mobility in soils. The same authors drew attention towards the study of MC persistence in agricultural soils, working with soils mimicking field moisture regime. They estimated half-times for the different chemical species and observed that these half-times were highest for MC-RR, which is supposed to be adsorbed more easily, and biodegradation, not adsorption, was identified as major dissipation process.

Thus, in our opinion MC in the sediments may not be related to adsorption, but to persistence of settled MC-producing organisms. On the one hand, according to our data, adsorption of free MC to sediments may most likely not taking place to great

extent. On the other hand, field data are available suggesting persistence of producing organisms and linking their presence with MC in the sediments. Massive presence of potential MC producers at the sediment surface has been described by Ihle et al. (2005) or by Mohamed et al. (2007), who observed that MC concentration correlated with the occurrence of *Microcystis aeruginosa* in the sediments. Welker et al (2007) were able to demostrate that quimiotypes of *Microcystis* cells were stable in benthic and pelagic samples, thus suggesting high integrity of *Microcystis* cells during the benthic stage. Also, our previous data (Wörmer et al., submitted) described sedimentation of intact MC-producing organisms being responsible for MC sedimentation rates of up to 2.53 mg g^{-1}. Latour et al. (2007) were able to detect high concentration of *Microcystis aeruginosa* in sediment depths of up to 70 cm. Therefore, we suggest that MC might be present in sediments, but mainly inside settled organisms. Still, in order to clarify this aspect, experiments including the very specific conditions in the sediments, as high humidity, variable presence of organic carbon, longer exposure time, oxygen deficiency or possible wetting-drying cycles should be conducted.

Proving of such hypothesis would require the existence of effective and reliable extraction methods. As said before, diverse methods have been suggested for example by Babica et al. (2006) and Chen et al (2006a). Still, in our study, recovery of spiked MC and detection of naturally occurring MC by these methods was not satisfactory. The only method that was able to detect MC presence in our sediments relied on previous separation of cyanobacteria by density-gradient centrifugation. The MC concentration obtained by our analysis is 0.409 µg g^{-1}, which is similar to concentrations described by Tsuji et al (2001) ranging from 0.08 to 2.33 µg g^{-1}, Chen et al. (2006a) (0.18 to 2.52 µg g^{-1}) or Babica et al. (2006) (0.016 to 0.474 µg g^{-1}), each using different extraction and/or detection methods. The MC concentration found in our studies has to be attributed solely to cell-bound fraction, as it was extracted from *Microcystis* colonies retrieved by density-gradient centrifugation. This fact again points towards the importance of intracellular toxin in the sediments, in contrast to possible adsorption procedures.

It is remarkable that it was necessary to physically separate the MC-containing particles from the rest of the sediment in order to detect and quantify the toxin. This indicates that possibly our failure in detecting MC by other methods has to be explained partly by means of poor detection due to interfering compounds extracted from the

sediments along with the toxin. In this way, recently Schmidtkunz et al. (2009) described the benefits of size exclusion chromatography cleanup on the HPLC-PDA detection of MC in sediments. The authors clearly show that size exclusion is able to improve detection, while reminding that other techniques, as for example the more common C18 solid phase extraction, may not be useful due to the wide range of MC hydrophobicity (Hummert et al., 1999). Further, they hypothesize that most of the failure in extracting MC from natural sediments and MC-spiked freeze-dried sediments may be at least partially explained by the problems caused by the great number of extractable UV active substances in sediments, which interfere with correct chromatographic separation and detection. Therefore, enrichment of MC due to size exclusion or selection of MC producers by gradient centrifugation may be mandatory in order to allow correct detection and quantification.

Sediments have been suggested as important sinks for MC, and the fate of these toxins in the sediment is of greatest concern. Our data and the available literature suggest that even if clay has great adsorption potential, in the field such adsorption may be limited by the very specific conditions found in lake and reservoir sediments. Further studies might therefore focus more strongly of the MC caged inside settled producing organisms. Another goal would be the development of an effective extraction method for MC. In our opinion, such methods should consider both the strong potential adsorption to clay and the need for active selection of MC containing organisms or of MC in the extract.

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7. Conlusiones / Conclusions

1. Las cianobacterias son un componente fundamental de las comunidades fitoplanctónicas en embalses españoles, siendo su época de máxima presencia aquella comprendida entre los meses de agosto y septiembre en cuencas más meridionales y entre septiembre y octubre en las más septentrionales.

1. Cyanobacteria suppose one of the major components of phytoplanktonic communities in Spanish reservoirs. Their presence is highest from August to September in the Southern watersheds and from September to October in the Northern basins.

2. Estas cianobacterias son responsables de la presencia habitual de microcistinas, las cuales han sido detectadas en la mitad de los sistemas analizados. También se ha confirmado la existencia en aguas españolas de cilindrospermopsina y anatoxina-a, esta última en muy bajas concentraciones. Estos datos, y especialmente la amplísima distribución de microcistinas y la novedosa aparición de la cilindrospermopsina, identifican a las cianotoxinas como un elemento clave en la gestión del recurso hídrico.

2. These cyanobacteria are responsible for the recurrent presence of microcystins, which have been detected in half of the tested reservoirs. Cylindrospermopsin and anatoxin-a have also been detected, allthough the latter one in only very small concentration. The wide distribution of microcystins and the novel and apparently increasing presence of cylindrospermopsin clearly identify cyanobacterial toxins as a key factor in the management of water quality.

3. Mediante la elección de una combinación acidificada de diclorometano y metanol como solvente y la preparación previa de la muestra, se ha podido optimizar un método de extracción en fase sólida para la recuperación de la cilindrospermopsina de matrices acuosas. La viabilidad de esta técnica se ha comprobado para un amplísimo rango de carga de cilindrospermopsina, así como en aguas naturales y en muestras enriquecidas con fuentes de carbono orgánico disuelto.

3. The use of an acidified mixture of dichloromethane and methanos as solvent and previous sample preparation did allow establishing an effective solid phase extraction method for cylindrospermopsin. Excellent response over a wide range of CYN and DOC loadings, as well as in natural samples, could be demonstrated.

4. Se ha demostrado la eficiente degradación *in situ* de las microcistinas, mediante las vías de la bio- y fotodegradación. La biodegradación actúa a gran velocidad, consumiendo entre un 13 y un 19% de la toxina añadida cada día. Sin embargo, también observamos fases lag de cerca de una semana, las cuales se explican por la necesidad de selección competitiva de bacterias degradadoras de microcistinas y/o por el consumo preferencial de otros compuestos disponibles. A su vez, sugerimos la existencia de rutas de biodegradación distintas a las ya descritas en otras zonas geográficas, una hipótesis que sería de relevancia a la hora de desarrollar sondas para la detección de la potencial capacidad degradadora de microcistinas. La fotodegradación – debida en campo a radiación UV-A y PAR – puede ser efectiva en sistemas poco profundos o en capas de mezcla de escaso espesor. Sin embargo, en sistemas más profundos, la reducida penetración de la irradiancia en la columna de agua dificultará dicha degradación.

4. Microcystins are efficiently degraded by both bio- and photodegradation. Biodegradation may be acting at high rates, between 13 and 19% of toxin being consumed each day. Nevertheless, lag phases of about one week were also observed, and explained by the presence of competing substrates and/or the need for competitive selection of microcystin degrading bacteria. Further, considering the degradation pathways describedso far, our results may suggest the existence of alternative gene clusters and enzimatic pathways. Such findings would be of greatest interest when designing genetic probes for rapid detection of potential microcystin degrading bacteria. Photodegradation of microcystins is carried out mainly by UV-A and PAR and may be effective in shallow systems or thin mixed layers, where vertical attenuation of incoming radiation is not as important.

5. Nuestros datos demuestran que un sumidero igualmente importante de microcistina puede ser la sedimentación de organismos productores de dicha toxina. Hemos podido estimar que en cualquier momento del desarrollo del bloom, entorno a un 4,5% de la toxina en fracción sestónica se ve sometida a procesos de sedimentación. Esto se traduce en una constante y masiva deposición de toxina, responsable de que cada día puedan sedimentar incluso varios miligramos de toxina por metro cuadrado.

5. Our field data demonstrate that sedimentation of more or less intact microcystin containing organisms may be a very important sink for this toxin. We estimated that, during the bloom period, about 4.5% of sestonic toxin is settling at any given time-point. This fact translates in a continuos and massive deposition of microcystins: each day, several miligrams of toxin may be settling per square meter.

6. La microcistina es fuertemente adsorbida por arcilla y por algunos sedimentos secos o combustionados. Sin embargo, nuestros resultados ponen en duda la viabilidad de dicha adsorción en las condiciones encontradas en el sedimento de un embalse, al verse fuertemente limitada por la presencia de agua y/u otros componentes orgánicos. Esto se traduce en que la microcistina en sedimento puede estar, en principio, disponible para procesos de degradación liberación a la fase acuosa.

6. Microcystins are strongly adsorbed to clay and to some sediments when these are dried or mineralized. Still, according to our results, we keep some reservation concering the possibility of adsorption actually occurring in the field, as the presence of both water and other organic compounds seems to stronly limit these sorption processes. This means that microcystins in the sediment may, in principle be available for degradation or liberation to the aqueos phase.

7. La correcta cuantificación de microcistinas en sedimento no ha podido ser lograda, dada la poca fiabilidad de las técnicas de extracción y cuantificación disponibles. Considerando los resultados obtenidos, sugerimos que los repetidos fracasos en cuanto al desarrollo de una metodología adecuada se deben principalmente a problemas de detección, y no de extracción. La separación física de la célula que contenga la toxina por centrifugación en gradiente o de la toxina una vez extraída parece solucionar en buena medida estos problemas.

7. Correct quantification of microcystins in the sediment could not be achieved. In our opinion, such failure may be mainly explained by problems during HPLC-PDA detection, and not during extraction. Physical separation of producing organisms by centrifugation or the extracted toxin by size exclusion seem to be somehow solving these problems.

8. La respuesta de la cilindrospermopsina a los potenciales agentes degradadores es radicalmente distinta a la de la microcistina. De hecho, bajo las condiciones experimentales empleadas, no pudo observarse su degradación por vía biológica en un plazo de 40 días, independientemente de la previa exposición de la población bacteriana a la toxina. Posiblemente la presencia de otros compuestos orgánicos más fácilmente degradables resultan esenciales a la hora de explicar esta lentísima degradación. En cuanto a la fotodegradación, ésta es igualmente ineficaz, requiere aparentemenente elevadísimas concentraciones de fotosensibilizadores y se debe casi exclusivamente a la radiación ultravioleta. Por otra parte, se ha demostrado la escasa tendencia de esta toxina a ser adsorbida por sedimentos o arcillas. Todos estos factores contribuirán a las altas concentraciones de cilindrospermopsina extracelular observadas en campo.

8. Cylindrospermopsin behaves differently than microcystins. Biodegradation is very strongly limited and, under the conditions tested, did not take place in a 40 day period, even when the bacterial community was previously exposed to the toxin in the field. Availability of more easily degradable C sources may be an explanation for the absence of biodegradation observed. Photodegradation, too, seems inefficient in the field, as it is apparently demanding very high concentration of photosensitizers and is mediated almost only by UV radiation. Finally, we were able to demonstrate that cylindrospermopsin is not adsorbed neither to tested sediments nor to clay. All these aspects may be strongly contributing to the high amounts of cylindrospermopsin found in the extracellular phase in field samples.

9. La distinta eficacia de la degradación, la tendencia de la microcistina a la sedimentación y la importante excreción de cilindrospermopsina explican la muy dispar distribución de ambas toxinas. La masiva presencia de cilindrospermopsina en fracción disuelta, así como la posible acumulación de microcistinas en sedimento, exigen una especial atención por parte de científicos y gestores.

9. The different efficiency of degradation mechanisms, the high sedimentation of microcystins and the strong liberation of cylindrospermopsin from producing organisms may be contributing to the very different distribution of both toxins. The massive presence of dissolved cylindrospermopsin and the probable accumulation of microcystins in the sediment demand special care from both scientists and water management.

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